Proceedings of the Meeting

“Fundamental and Practical Approaches to Increase Biocontrol Efficacy”

at

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Edited by

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Preface

The meeting of the IOBC/WPRS working group ‘Biological control of Fungal and Bacterial Plant Pathogens’ was held in Sol Cress Centre, Spa, Belgium, on September 6-10, 2006. It was entitled 'Fundamental and practical approaches to increase biocontrol efficacy'.

During the meeting the following themes were emphasized:
1. Biocontrol in various systems;
2. Understanding/stimulating naturally occurring antagonists in soil, substrate, rhizosphere and phyllosphere;
3. Understanding/stimulating the modes of action and ecological suitability of introduced antagonists in soil, substrate, rhizosphere and phyllosphere;
4. Influence of plant species/cultivar on natural or introduced antagonists;
5. Production and formulation to increase biocontrol efficacy;
6. Use of biocontrol agents in integrated disease management;
7. Risk assessment.

A large number of participants (154) from 30 countries attended the meeting. Discussions among plant pathologists and other plant protection experts were held while the audience included many students and technicians as well. The role of mode of action studies in understanding how biocontrol agents are inflicting their effects and in improving their activity was emphasized. The need for more work on formulation and biocontrol enhancement including integration of control methods for real life management of diseases was widely expressed. A call for more research on these subjects was raised. A concern was expressed regarding the difficulties in commercialization of microbial biocontrol and other alternatives and the delay in implementation of friendly means of control.

We wish all of us fruitful, interesting and enjoyable meetings also in the future. We acknowledge the help of Mr. Damien Friel in collecting the manuscripts of this volume and of students from the Department of Crop Protection, Laboratory of Phytopathology, Ghent University and from the Plant Pathology Unit, Gembloux Agricultural University, Belgium for their assistance before, during and after the meeting.

Yigal Elad, Convenor of the ‘Phytopathogens’ IOBC/WPRS WG

The editors of the present volume of the bulletin wish to thank all the contributors for summarizing their works. The contributions present valuable information for the science and implementation of efficient biocontrol agents of plant diseases.

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http://www.iobc-wprs.org/structure/index.html  
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Spa, Belgium, September 6-10, 2006 meeting

Fundamental and Practical Approaches to Increase Biocontrol Efficacy

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Biocontrol in various systems
Is it possible to improve biocontrol efficacy in some plant/pathogen systems?

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Abstract: The use of microorganisms in plant disease biocontrol is, in theory, a powerful alternative to chemical pesticides. Many microorganisms are constantly selected by researchers for their antagonistic activity in in vitro or in planta screening systems, but only few of them eventually show a good and consistent efficacy in field experiments. One of the main reasons for not reaching commercialisation can be related to markets that are financially too small to justify development and/or registration costs. Even when a commercial biocontrol agents (BCA) based product is available, successful use of the product is hampered by inconsistency. In spite of intensive research efforts, some diseases are difficult to be controlled with BCAs. But why biocontrol is more likely to be successful in some patho-systems than others? Monocyclic diseases or diseases with a limited and predictable window of infection opportunity, slow progress rates or where a certain level of damages is acceptable have more chances to be controlled with BCAs. BCAs do not easy control pathogens that quickly penetrate and develop into host tissues (i.e. Plasmopara viticola) or produce resistant and persistent structures (i.e. Armillaria mellea). Biocontrol extent may depend on environmental parameters. Some BCAs are active only in a narrow range of temperature and relative humidity. Most of them last for a short time after application because they are susceptible to UV light, drought, extreme temperatures or to microbial community that colonise the environment or simply because they do not find a suitable growth substrate. Gaining knowledge on the limiting environmental parameters and the biocontrol mechanism and obtaining a good formulation, bust also selecting the suitable plant pathogen system can help increasing the efficacy in field.

Key words: biocontrol agent, Plasmopara viticola, Armillaria mellea, Podosphaera aphanis

Introduction

The use of microorganisms in plant disease biocontrol is, in theory, a powerful alternative to chemical pesticides. Although in the last 25 years research in plant pathogen biocontrol has increased dramatically and the number of patents of biocontrol related products indicates a potential commercial interest (Fig. 1), only a small part of the total sales of fungicides is represented by biocontrol agents (BCAs). More than 1200 scientific papers dealing with microbial biocontrol of plant pathogens have been published between 2001 and 2006, demonstrating that lots of efforts and resources were allocated on this topic. Quite a lot of microorganisms are constantly selected by researchers for their antagonistic activity in vitro or in planta screening, but only few of them show afterwards a good and consistent efficacy in field experiments. Several BCAs, after a first characterization are not patented or commercially developed. When this paper was written the microbial strains listed by EPA as biopesticide active ingredients were 38, with this number decreasing to 27 when considering the different species and not the specific strains. Only 22 out of 38 active ingredients are currently on the market (corresponding to about 220 commercial products).
One of the main reasons for not reaching commercialization can be related to a financially too small market (BCAs are often highly specific against one or few pathogens) to justify development and/or registration costs. The proof for this hypothesis can be found in the higher number of active ingredients registered in USA (38), compared to Europe (6 active ingredients in Annex I) where the registration process is longer, more expensive and difficult. The cost of registration in Europe is probably the principal obstacle to the development of new products. BCAs are subject to prohibitive registration fees and extremely high costs for providing data (Directive 91/414/EEC) and only more simple procedures may permit the economical development of new products. In addition the structure of the global market of BCAs is significantly different from chemical pesticide industry: BCAs manufacturing companies are small and numerous if compared to chemical ones where 90% of market is in the hands of seven multinational companies. The intrinsic costs of BCAs are also higher compared to chemical pesticides because, being living organisms, they have a short shelf life and complex production process. In conclusion the high cost of development, registration and production of a BCA and the small profits make BCAs development a risky business attracting only a modest number of investors. In addition, most of the leading agrochemical companies that had undertaken research on BCAs in the past, have disinvested this business in favour of the potential higher returns of genetically modified crops.

In general BCAs are less effective than chemicals and they need to be integrated with other control agents. In practice it results in higher risk of losses and their efficacy must to be confirmed in every new environment. Since they are often highly influenced by environmental factors, wide knowledge on their mechanism of action and high technical skills are required for a successful use. Their application is often complicate and a continuous monitoring of the crop and diseases are commonly needed, thus resulting in additional costs.

BCAs can successfully compete on the market against chemical fungicides only in particular situations (in organic farming where no chemical inputs are permitted and in integrated crop management when a reduction of chemical pesticides is sought). Even when a commercial BCA is available and competitiveness on the market is present, the successful use of the product is hampered by inconsistency. Efficacy and consistency can be improved acting on several aspects: survival of the microorganism, actions on the pathogen and on the plant. BCA survival can be prolonged using suitable formulation, but knowledge on this topic is scarce and often extrapolated from pesticide industry. Repeated application increase costs and applying in condition suitable environmental for the BCA may be impossible.
Microbial and chemical partners in the formulation to complete the effect must be harmless for the BCA. Theoretically it is possible to act to reduce competition with existing microflora, yet practically very difficult. To accomplish the best effect of the BCAs the right time for application (i.e. when the pathogen is more susceptible) must be chosen and it can be integrated with chemicals in an integrated pest management program. The use of BCAs inducing resistance of plants and the application on plant stages with low susceptibility to disease can increase the percentage of success.

In spite of the availability of an effective microbial antagonist and application of measures to improve its efficacy, some diseases are still difficult to be controlled with BCAs in the field. Are there specific reasons why biocontrol is more likely to be successful in some pathosystems than in others? Monocyclic diseases or diseases with a limited and predictable window of infection opportunity, slow progress rates or where a certain level of damages can be accepted have more chances to be controlled with BCAs.

Crops which products are transformed are in general more suitable compared to crops for the fresh market: one percent of severity of *Plasmopara viticola* on a grapevine cluster has not the same economical impact of the same percentage of scab on apples or grey mould on strawberry. One spot of apple scab implies that the fruit is lost for the market and one infected strawberry in a box can rapidly affect all the fruits during storage or selling. With scenarios based on 2003 prices and production in Trentino region (northern Italy) it can be estimated that the economical impact of 1% of incidence with 1 and 10% of severity on grapevine cluster is respectively 13 and 127 €/ha, while the same level of disease on apples gives respectively a lost of 1542 €/ha irrespective of the severity. BCAs do not effectively control pathogens that quickly penetrate and develop into host tissues (i.e. *P. viticola*) or produce resistant and persistent structures (i.e. rhizomorphs of *Armillaria mellea*).

Biocontrol efficiency may depend on environmental parameters. Some BCAs (i.e. *Ampelomyces quisqualis*) are active only in a narrow range of temperature and relative humidity. Most of them last for a short time after application because they are susceptible to UV light, drought, extreme temperatures or to microbial community that colonize the environment or simply because they do not find a suitable substrate for growth. Selecting BCAs according the limiting environmental parameters, mechanism of action and long survival can help in increasing efficacy in field.

Three pathosystems representing major typologies, *P. viticola*/grapevine, *A. mellea*/ grapevine and *Sphaerotheca macularis*/strawberry, will be discussed regarding suitability for biocontrol, efficiency in discovering new BCAs, market positioning, growers needs and chemical competitors.

**Plasmopara viticola*/grapevine**

In spite of European restrictions on the use of copper in organic agriculture (Commission Regulation EC No 473/2002 amending Council Regulation EEC No 2092/91), the consequent efforts for finding BCAs against *P. viticola* and the fact that grapevine downy mildew is a destructive disease in warm and wet climate there are no commercial BCAs available at the moment against this pathogen. Comparing the efficacy of BCAs in controlling *P. viticola* under controlled conditions on leaf disks with greenhouse experiments on the whole plant there is in general a dramatic decrease (Fig. 2) and if we compare the efficacy against artificial infections under greenhouse controlled conditions and the efficacy obtained under natural field conditions the situation is even worse (Fig. 3) (Vecchione et al., 2004).
Figure 2. Efficacy of several fungal antagonists against *P. viticola*: comparison between laboratory (leaf disk test) and greenhouse (whole plant) experiments. BCAs water suspensions (10⁶ conidia/ml) were applied immediately before pathogen artificial inoculation (10⁵ sporangia/ml). Leaf disks and plants were maintained under controlled conditions (20°C, 80±10% relative humidity). Efficacy was calculated of percentage of disease severity (symptomatic leaf surface) reduction.

Figure 3. Efficacy of commercial microbial antagonists against *P. viticola*: efficacy in 10 greenhouse and 3 field trials using the commercial products: TRICHODEX (*Trichoderma harzianum*), Clonotri (*Clonostachis rosea + T. harzianum*), Serenade (*Bacillus subtilis*). BCAs were applied once at commercial dosages before *P. viticola* artificial inoculation (in greenhouse) and under natural infection conditions (field). Assessments were done at the end of incubation in greenhouse experiments and at harvest time in field experiments.

The decrease in efficacy can be related to several factors including a poor survival, reduced activity, competition with natural microflora, uneven distribution and, in case of polycyclic diseases as grapevine downy mildew to the sum of uncontrolled infections at each infection time. In addition *P. viticola* sporulates only under suitable conditions (end of incubation period, dark, water, temperature, etc.), zoospores need water in order to reach the stomata and start the infection. After penetration *P. viticola* grows inside the tissues and only after the incubation period, new sporangia exit from stomata to start others secondary cycles. *P. viticola* infections occur in a narrow range of weather conditions (suitable temperature,
presence of water) and if stomata are differentiated, but when *P. viticola* penetrates into leaf tissues, it cannot be controlled without a curative active ingredient. Additionally even if sporulation is reduced, new infections from oospores arise under suitable conditions.

The time between sporangia germination and penetration (time in which the pathogen can be easily killed) is few hours (even in optimal conditions), therefore resulting in a short time for control action and it is almost always during rain, which make a timely BCAs treatment very difficult. Rain and leaf wetness forecasts are not too reliable therefore downy mildew control is based on preventing pathogen penetration, by continuously protecting leaves and bunches with long lasting toxic compounds. In general, if they are not curative, control agents against downy mildew should have a long residual effect and are applied as preventive treatments. Since BCAs are not surviving for long periods on the phylloplane and they are not as fast and effective as chemical fungicides, biocontrol of downy mildew seems to be very hard to achieve.

Necrosis appears soon after the infection on leaves and bunches. Theoretically a single zoospore can cause loss of the entire bunch and billions of sporangia are produced in the vineyard. During bloom and until the bunch reach low disease susceptibility, BCAs (or poorly effective control agents) are not suitable. A reasonable used of BCAs can be the application during period of low infection risk and always in integration with chemicals.

**Armillaria mellea/grapevine**

*A. mellea* is the causal agent of root rot on grapevine. The pathogen can survive for long periods (up to 5-10 years) in the roots and woody residues in the soil. Chemical pesticides are ineffective in controlling the disease, mainly because the long inoculum persistence and the difficulty to kill the pathogen inside the dead woody material. In this situation an effective BCA can have high probability of market success, but BCAs in general survive for a short period after application.

![](image.png)

Figure 4. Percentage of effective microorganisms against *Armillaria mellea* in dual culture, wood pieces and on grapevine plants, when isolated from phyllosphere, rizosphere and soil (a total of 245) (left, Pertot et al., 2002) and isolated from decaying *A. mellea* fruiting bodies (a total of 109) (right, Pertot et al., 2006)
An isolation system that enhances the probability of finding the appropriate microorganism may improve the percentage of success (Fig. 4). The number of BCAs that are found effective on plants against *A. mellea* is higher when microorganisms growing on *A. mellea* fruiting bodies are isolated compared to a traditional isolation method (Pertot et al., 2002, 2006). The high number of effective BCAs could be related to the better survival in soil in the presence of the pathogen or the specific mechanism of action (hyperparasites, for example).

**Podosphaera aphanis/strawberry**

Powdery mildew of strawberry caused by *P. aphanis* is a dangerous disease in Mediterranean climate and in plasticulture. The strict pesticide schedule needed for its control raises the problem of high chemical residues on fruits, sometimes exceeding the maximum residue levels (MRL). *P. aphanis* is an ectoparasite, with all elements on the leaf surface except the haustoria and can be killed (or strongly reduced) even after haustoria penetration. The period between conidia germination and haustoria formation (fungus susceptible to any adverse conditions) is longer, for example, than the grapevine downy mildew and there is more time for control activity. Powdery mildew slowly reduces functionality of tissues. In addition if a good disease control is obtained at the beginning of the season (until fruit set), since old leaves are less susceptible, the epidemic slowly increases even with less effective control agents. Powdery mildew continuously produces conidia and infection conditions are almost daily given (high relative humidity), but timing of the fungicide application is less relevant then for other pathogen. It is important that the BCA can kill mycelium and conidia, therefore products that can constantly reduce the fungus on leaf surface or parasitize the reproductive structure (i.e. *A. quisqualis*) can successfully control the diseases.

![Graph showing severity of powdery mildew on strawberry leaves with different strategies](image)

Figure 5. Severity of powdery mildew on strawberry leaves with different strategies: fully chemical (CHEM1), integration of BCAs and chemicals (COMB1, COMB2, COMB3) and fully BCAs based (TRICHO, AQ10, Serenade). Treatments were weekly applied on strawberry on raised beds in plastic tunnels in northern Italy (partially modified from Pertot et al., 2004).

Powdery mildew of strawberry is in general more suitable for biocontrol especially if the more susceptible stages of the disease are controlled with a chemical fungicide. In fact also in this case when BCAs are used all along the season are poorly effective (Fig. 5), but when
integrated with early applications of chemicals, a relevant reduction of residues and same control level as chemicals are achieved (Pertot et al., 2004).

Conclusions

From the industry point of view one of the key points is the convenience of the research and development process: cost of development, patent and registration of a BCA has to be compensated by a commercial success. A frequent approach is to estimate the potential market in term of hectares or production, estimate the market of the chemical fungicides related to the crop and single pathogen and then look for a suitable BCAs. This approach tends to promote the development of BCAs against the most relevant diseases in the most important crops, where the chemical competitors are equally strong, or of BCAs with a wide spectrum of activity penalizing very effective but specific strains and risks to focus on plant/pathogen system not suitable for biocontrol.

Most of the studies on biocontrol focus on the behaviour of the microbial antagonists after isolations and screening and suitability of the pathogen to be controlled at a satisfying economical threshold by a BCA has received little attention. A substantial progress could be made in the future if the biological characteristics of the pathogen and its epidemiology are taken in account before starting the selection of potential BCAs. Isolation methods based on specific function, metabolite, and conditions and the possibility of integration with chemical fungicides may improve the number of commercially successful BCA.

Acknowledgements

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References


Development of biocontrol of powdery mildew diseases

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Abstract: Powdery mildews are important targets for the development of control agents. We developed biocontrol systems that are capable of controlling various powdery mildew pathogens, including those of grape, cucumber, zucchini, tomato, pepper, barley and strawberry. Bacteria, yeasts, and filamentous fungi originally isolated from the canopy of wild and cultivated plants were collected and sprayed on plants before challenge by the respective powdery mildew pathogen. Activity was related to induced resistance and change of populations of indigenous microflora and/or inhibitory compounds. The purpose of the research was to improve powdery mildew control. Supplements to the cell suspension and increased frequency of applications resulted in higher population levels of the biocontrol agent on plant surfaces and resulted in better suppression of powdery mildew diseases. One yeast was sprayed at the rate of $10^7$ twice a week in grape vineyard cv. Carignaen whereas the chemical fungicides Kresoxim methyl (Stroby at 0.02%) and Triadimenol (Shavit at 0.01%) were sprayed as recommended. Incidence of powdery mildew reached more than 90% twenty three days after initiation of the experiment and in plots of the two chemical treatments and the biocontrol treatment it was 0-15%. Three weeks later (four weeks after the last treatment) disease slightly increased and was c. 25% in the biocontrol plots and significantly less in the chemically treated plots. The severity of powdery mildew reached c. 70% in the untreated control whereas the different treatments showed minor symptoms. It is concluded that the biocontrol system can potentially serve as a management tool for powdery mildews.

Key Words: Erysiphe Necator, Oidium neolycopersicum, Podosphaera xanthii, Sphaerotheca macularis

Introduction

Powdery mildews are important targets for the development of control agents. Most of the microorganisms reported as BCAs of powdery mildew fungi are hyperparasites or antibiotic producers (Bélanger et al., 1997; Elad et al., 1995, 1999). The most common hyperparasite of powdery mildews is the coelomycete, Ampelomyces quisqualis (Sztejnberg et al., 1989). The species does not appear to be confined to one host since, for instance, an isolate from an Oidium sp. infecting Catha edulis in Israel could antagonize several powdery mildew fungi belonging to the genera Oidium, Erysiphe, Sphaerotheca, Podosphaera, Uncinula and Leveillula (Sztejnberg et al., 1989). Other isolates parasitized Sphaerotheca fuliginea in cucumbers (Jarvis & Slingsby, 1977; Philipp & Crüger, 1979; Sundheim & Amundsen, 1982; Sztejnberg et al., 1989). The hyperparasite Verticillium lecanii of several pathogenic fungi reduced the incidence of Sphaerotheca fusca in greenhouse when certain conditions were respected, namely low vapour pressure deficit (VPD) (Spencer & Ebben, 1983; Verhaar et al., 1996). Considered for a long time a strict hyperparasite, recent findings have suggested that early degradation of powdery mildew cells in interaction with Verticillium lecanii was
mediated by the production of antibiotic substances (Askary et al., 1997). Among other fungi reported as parasites are *Acremonium alternatum* and *Cladosporium cladosporioides* that antagonized and destroyed the thallus of *Sphaerotheca fusca* (Malathrakis & Klironomou, 1992).

The antagonistic epiphytic yeast-like fungus *Sporothrix flocculosa*, colonized powdery mildew of rose and cucumber more rapidly than *Sporothrix rugulosa*; *Sporothrix flocculosa* did not penetrate its host but rather induced a rapid plasmolysis of the powdery mildew cells (Hailaoui et al., 1992; Hailaoui et al., 1994), presumably the result of the production of fatty acids by the antagonist (Benyagoub et al., 1996). The fatty acids appear to interfere with the integrity of the plasmalemma, the composition of which determines the specificity of the antagonist. Hoch & Providenti (1979) found strong antagonism between the common phyllosphere yeasts, *Tilletiopsis* spp. and the cucumber powdery mildew pathogen. Inoculation with a *Tilletiopsis*-cell suspension on detached, mildew-infected cucumber leaves, destroyed the superficial thallus of the powdery mildew. Similar to *Sporothrix flocculosa*, *Tilletiopsis* spp. do not seem to penetrate the powdery mildew fungus suggesting that antibiosis is the main mode of action (Klecan et al., 1990).

The purpose of the research was to improve powdery mildew control. For this we tested several microorganisms for their ability to control powdery mildews. We attempted to develop means to improve efficacy and studied biocontrol under field conditions.

**Materials and methods**

Bacteria, yeasts, and filamentous fungi originally isolated from the canopy of wild and cultivated plants were grown in liquid culture, collected and sprayed with a hand sprayer on plants before challenge by the respective powdery mildew pathogen. Disease severity was evaluated by visually estimating the area of plant surfaces covered by typical powdery mildew symptoms. Disease progress curves were drawn and the area under curves were calculated.

Population of treated microorganisms on leaf surfaces were evaluated at given times after application by agitating the leaves in water supplemented with 0.01% Tween 80, ten-fold serial dilutions and placing 10 µl drops on solid medium in 90 cm diameter Petri.

**Results**

We developed biocontrol systems that are capable of controlling various powdery mildew pathogens, including those of grape, cucumber, zucchini, tomato, pepper, barley and strawberry.

**Experiments under controlled conditions**

Cucumber and tomato plants treated by the various biocontrol agents showed significantly lower disease severity as compared with the untreated control. On tomato plants, sulfur did not suppress disease better than the biocontrol agents and *T. harzianum* T39 was found similar to the different biocontrol agents and to sulfur (Table 1).

Application of three yeast biocontrol agents on cucumber leaves significantly suppressed disease. Moreover, application at higher frequency (twice a week instead of one every week) resulted in better disease suppression (Table 2).
Table 1. Control of *Podosphaera xanthii* on cucumber and *Oidium neolycopersici* on tomato by selected control agents during 27 days after application. Results are presented as area under disease progress curve (AUDPC, % cover severity×days)

<table>
<thead>
<tr>
<th>Control agent</th>
<th>Cucumber</th>
<th>Tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>211 a</td>
<td>370 a</td>
</tr>
<tr>
<td>B69</td>
<td>152 b</td>
<td>132 c</td>
</tr>
<tr>
<td>B71</td>
<td>138 b</td>
<td>140 c</td>
</tr>
<tr>
<td>Y13</td>
<td>129 b</td>
<td>108 c</td>
</tr>
<tr>
<td>Y89</td>
<td>174 ab</td>
<td>nt</td>
</tr>
<tr>
<td>Y16</td>
<td>45 c</td>
<td>203 b</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em> T39</td>
<td>nt</td>
<td>150 bc</td>
</tr>
<tr>
<td>Sulfur</td>
<td>nt</td>
<td>230 b</td>
</tr>
</tbody>
</table>

nt=not tested

Numbers in each column followed by a common letter are significantly not different (*P*≤0.05).

Table 2. Control of *Podosphaera xanthii* on cucumber by weekly and bi-weekly application of three biocontrol agents during 25 days after application. Results are presented as area under disease progress curve (AUDPC, % cover severity×days)

<table>
<thead>
<tr>
<th>Control agent</th>
<th>Weekly</th>
<th>Bi-weekly</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>255 a</td>
<td></td>
</tr>
<tr>
<td>Y13</td>
<td>187 b</td>
<td>124 c</td>
</tr>
<tr>
<td>Y89</td>
<td>182 b</td>
<td>97 c</td>
</tr>
<tr>
<td>Y16</td>
<td>152 b</td>
<td>98 c</td>
</tr>
</tbody>
</table>

Numbers followed by a common letter are significantly not different (*P*<0.05).

The efficacy of a biocontrol (BCA) agent is affected by its ability to survive on target plant surfaces for extended periods of time. We tested the survival of cells of one yeast isolate (Y16) on strawberry leaves and found that it survived best on strawberry leaves when it was applied in solution with a particular set of additives. Surprisingly, glucose did not affect the survival of the yeast (Table 3).

Table 3. Population (no./cm²) of the biocontrol agent Y16 on strawberry leaves. The yeast suspension was applied at 1.7×10⁶/cm² was supplemented with glucose and formulation additives

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>No additive</td>
<td>3.2×10⁵</td>
</tr>
<tr>
<td>+ glucose</td>
<td>3.9×10⁵</td>
</tr>
<tr>
<td>+ formulation</td>
<td>1.7×10⁶</td>
</tr>
<tr>
<td>+ glucose + formulation</td>
<td>3.7×10⁶</td>
</tr>
</tbody>
</table>

Numbers followed by a common letter are significantly not different (*P*<0.05).

These supplements also affect general indigenous microorganism populations. The level of natural bacteria populations decreased while natural yeast populations increased. When tested with several potential BCAs, the formulated spray solution successfully enhanced
control efficacy and survival of various microorganisms on strawberry leaves. Typically, population levels of the individual BCAs applied in the formulated solution were 150-300% higher than the populations of the same microorganisms applied as naked cells.

**Control under commercial conditions**

Y16 was chosen for further testing in a commercial cv. Carignane vineyard at the. It was compared with effective chemical fungicides in the vineyard during May-July, 2005. Y16 was sprayed at the rate of 10^7 twice a week whereas the chemical fungicides Kresoxim methyl (Stroby at 0.02%) and Triadimenol (Shavit at 0.01%) were sprayed 6 times. Incidence of powdery mildew reached more than 90% twenty three days after initiation of the experiment. The Incidence of infected bunches in plots of the two chemical treatments and the biocontrol treatment was 0-15% during six weeks after the initiation of the treatments. Three weeks later (four weeks after the last treatment) disease slightly increased and was c. 25% in the biocontrol plots and significantly less in the chemically treated plots. The severity of powdery mildew reached c. 70% in the untreated control whereas the different treatments showed minor symptoms.

**Discussion**

Several possibilities were found effective in improving control of the powdery mildews and ensuring higher populations of the biocontrol microorganisms on plant surfaces. These included choosing the right isolate for each plant-pathogen system, optimal cell concentration of the biocontrol agents, increasing frequency of application, mixtures of biocontrol agents and of combination of microorganisms with other control agents. Some of these were not presented in the results section.

We conclude that the biocontrol system can potentially serve as a management tool for powdery mildews. Activity was related to induced resistance and change of populations of indigenous microflora and/or inhibitory compounds.

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**References**


Evaluation of seed treatment methods for organic vegetable production

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**Abstract:** In organic farming, the availability of pathogen-free seeds in most crops is often hampered by a lack of effective non-chemical methods for sanitation of infested seed lots. In the framework of the EU-funded project STOVE "Seed Treatments for Organic Vegetable Production" (QLK5-2002-02239), currently available methods for control of seed-borne vegetable pathogens are investigated with the aim of further improvement, while in parallel new methods acceptable to organic farming are developed. The potential of three physical methods (hot water, hot humid air and electron treatment), of micro-organisms and different compounds of natural origin are investigated.

In greenhouse trials with carrot seeds highly infested with *A. dauci* and *A. radicina* the percentage healthy seedlings reached around 10% in the “untreated control”. Treatment with all three physical methods significantly increased the percentage of healthy seedlings up to 65%, while selected biological treatments also enhanced emergence and establishment of healthy seedlings, but to a lesser extent. The combination of hot water with seed application of an isolate of *Pseudomonas putida* resulted in an additive effect (61% healthy seedlings) compared to treatment with the single methods (appr. 42% for hot water and 24% for the bacteria). When these treatments were evaluated with the same seed in the field, the results showed the same tendency. However, the obvious additive effect of the combination treatment of hot water and *P. putida* could not be observed. Although an increase in the absolute number of plants per meter row was found for most treatments, the only significant difference to the untreated control was observed after seed treatment with hot humid air. Disease symptoms on carrot leaves due to *Alternaria* were in all treatments below 0.2%.

Apart from the results obtained with carrot seed infected with *Alternaria* spp., results from other pathosystems will be presented. For most pathosystems, the physical treatments resulted in a moderate to good control of the respective diseases. Also, from groups of treatment (microorganisms, plant extracts and inducers of resistance) candidates with promising control properties could be identified. The influence of the treatments on emergence was often more prominent under greenhouse than under field conditions.

The efficacy of given single and combined treatments depended strongly on the host / pathogen system investigated. Moreover, the seed lots differed to a large extent in sensitivity towards the physical treatments, so treatment parameters needed to be carefully adjusted for each vegetable species and seed lot. The presence of less mature seeds and the onset of germination processes prior to harvest were shown to be important factors increasing the sensitivity of seed lots to hot water and hot humid air treatments.
The lactoperoxidase system as a novel, natural fungicide for control of powdery mildew

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Abstract: A novel, natural fungicide-bactericide has been developed on the basis of the so-called LP-system (lactoperoxidase system), an anti-bacterial system active in bovine milk. The currently developed formulation is targeted to control powdery mildew in greenhouse vegetables and ornamentals and applied as a curative contact fungicide. Trials have been done in the Netherlands and in Spain in protected crops against powdery mildew in cucumber (Sphaerotheca fuliginea), in tomato (Oidium neolycopersici), in sweet pepper (Leveillula taurica) and in rose (S. pannosa) and showed effective control, between 85-98%. The product can be used in IPM programmes and is safe for natural enemies and pollinators. It is also compatible with a number of chemical fungicides, even in tank-mixes, and can become an important tool in resistance management programs.

Key words: bactericide, fungicide, greenhouse vegetables

Introduction

A novel, natural fungicide-bactericide has been developed on the basis of the lactoperoxidase system, an anti-microbial system active in milk. The enzyme lactoperoxidase and other heme-containing peroxidases fulfill an important role in the non-immune defense of higher organisms against microorganisms (Pruitt & Tenovuo, 1985). Together with hydrogen peroxide and a halide (I⁻) or the pseudohalide thiocyanate (SCN⁻), they form potent natural anti-microbial systems. The halide and thiocyanate can be oxidized by the peroxidases to reactive oxidizing species (ROS: e.g. IO⁻, SCNO⁻), which react with sulf-hydryl groups in proteins in microorganisms. The oxidized molecules are active against viruses, gram-positive and gram-negative bacteria, yeasts, fungi and probably also mycoplasmas.

Peroxidase systems are present in plants, animals and man and part of the non-immune defense against microorganisms, in mammals, mainly against bacteria. They can be found in body secretions such as saliva, tear fluid, bronchial, nasal and intestinal secretions and in milk. Bovine milk contains high concentrations of lacto-peroxidase. From this, the lactoperoxidase system or LP-system is derived. In milk the LP-system functions as a natural preservative agent and inhibits bacterial growth or kills them. The LP-system is used as a preservative in a number of food and non-food applications in dairy products, oral care products, and feed and veterinary specialties (Seifu et al., 2005).

The LP-system for disease control

Lactoperoxidase can be extracted from milk or whey in a highly purified form and can be used as a basic component in applications of the LP-system. The enzyme by itself has no activity, but needs a hydrogen peroxide source and a (pseudo) halide to be able to form the
reactive molecules. In milk thiocyanate (SCN⁻) is transformed into hypothiocyanite (SCNO⁻), a short-lived oxidation product that is active against bacteria.

Our research focused on developing a formulation with activity against fungal diseases, particularly powdery mildews. There were some indications that the addition of another halide, iodide, would enhance the activity of the LP-system against fungal organisms (Pruitt & Tenuvuo, 1985) and we found that at a certain ratio of thiocyanate and iodide the LP-system was able to kill fungal material, such as mycelium and conidia.

This has resulted in the formulation of a new H₂O₂ - SCN⁻ - HOS-R product for control of fungal diseases, called Enzicur. This formulation in which both KI and KSCN are incorporated, forming the active molecules HS-RIO⁻ and SCNO⁻, also has an activity against bacteria.

**Mode of action**

The mode of action is based on the oxidation of iodide and thiocyanate into the short-lived reactive oxygen species (ROS) hypothiocyanite (SCNO⁻) and hypoiodate (IO⁻). These react with sulfhydryl groups in proteins and the nucleotides NADH and NADPH. The oxidations of these compounds result in major changes in these molecules and loss of functions. The cytoplasmic membrane, sugar and amino acid transport systems, and glycolytic enzymes may be damaged. The result of such damage may be cell death or inhibition of growth, respiration, active transport, or other vital metabolic functions.

Most of the research on the activity of the LP-system has been carried out on bacteria and only some on fungi and yeasts. In the case of powdery mildew fungi it is not known how the system works in detail. We have seen that spores are killed (no germination anymore) and sometimes collapse, probably due to leakage. Also mycelium is killed and collapses. The effect of the ROS is a process of a few minutes to some hours depending on the exposure, dosage, and target organism. Direct contact between the ROS solution and the target is essential. The product shows a direct effect by contact and has no persistent or preventative effect, nor any systemic effect. When the water evaporates, the activity ceases.

**Control of fungal plant diseases**

The formulated product Enzicur has been tested primarily against powdery mildews in various crops. Resistance to chemical fungicides is a big problem and new solutions have to be developed to be able to obtain sufficient control. Powdery mildews form haustoria that are inside the plant tissue, the other structures of the fungus develop on the leaf surface. Therefore, contact between the ROS and the fungal material can be achieved. Fungal growth inside plant tissue or vessels is difficult to reach with the ROS. In order to improve the contact between the watery spray solution, containing the ROS, and the lipophilic mycelium an adjuvant based on vegetable oil has been developed. This adjuvant contains an emulsifier and a spreading agent and improves the contact and the efficacy.

Enzicur can be used as a curative control product and gives direct control, whereby mycelium and spores are killed and even disappear after 1-2 days. Control is as effective as with chemical fungicides. Examples of trials in cucumber and tomato are given in figure 1. The trials were done in commercial glasshouses and according to EPPO and GEP guidelines. Enzicur was compared with bitertanol (Baycor Flow) as a reference product. Two dosages of Enzicur were tested: 1.5 gr/l (1 N) and 3.0 gr/l (2 N); Baycor was tested in the recommended
rate of 0.6 ml/l. The applications were done on a naturally occurring powdery mildew infestation when leaves were covered for 10-20% with the disease. Assessments were made 2-3 and 7-9 days after spraying, assessing the percentage leaf surface covered with powdery mildew. In cucumber *Sphaerotheca fuliginea* was controlled up to 80% already after 2 days and to 95% after 8 days. The same was seen in tomato against *Oidium neolycopersici*. In sweet pepper, strawberry, rose and oak similar results have been achieved; Enzicur is able to control *Sphaerotheca* spp, *Erisyphe* spp, *Leveillula* spp and *Microsphaera* spp. Besides powdery mildew we have seen that this formulated LP-system can also kill *Botrytis*.

![Graph showing efficacy of Enzicur and Bitertanol on leaves of cucumber and tomato](image)

**Figure 1.** The effect of ENZICUR on powdery mildew (*Sphaerotheca fuliginea*) in cucumber (cv. NUN 2216) (left) and on powdery mildew (*Oidium neolycopersici*) in tomato (cv. Gratilda) (right) in the Netherlands in August 2003

**Compatibility with other control measures**

**Natural enemies and pollinators**

Enzicur and its adjuvant have been tested on a range of natural enemies that are used in greenhouse crops in IPM. Various life stages of the natural enemies have been exposed, either directly via a topical application or indirectly by putting them on freshly dried residue, sprayed on leaf discs. Mortality was assessed after 24 and 48 h and compared with untreated. Abbott corrected values were compared to the IOBC categories. The IOBC category was 1 (not harmful) for the following natural enemies: *Amblyseius cucumeris* (♀), *Amblyseius swirskii* (♀ and eggs), *Aphidius colemani* (♀ and mummies), *Encarsia formosa* (♀ and pupae), *Eretmocerus mundus* (♀), *Macrolophus caliginosus* (nimphs), *Orius laevigatius* (N3-4 nymphs). The product was only moderately harmful to young nymphs of *O. laevigatius*. Honey bees have been tested as part of the registration dossier according to the OECD guidelines No 213 and No 214 on oral and contact exposure. No harmful effects were found.

**Chemical pesticides**

We recommend not to tank-mix Enzicur with any other chemical insecticide and fungicide. Nevertheless growers try to minimise labour costs in treating their crops, so if possible they will spray tank-mixes. We have tested Enzicur with the most common greenhouse pesticides on compatibility. The active ingredients of Enzicur are enzymatically generated in the spray solution directly after loading. Possible negative effects of chemical products on the enzymatic activity could result in a non-active spray solution. Therefore it is recommended to prepare Enzicur first and then to mix another product in the tank 20-30 minutes later. Nevertheless the ROS could react with any chemical compound (active substance or formulant) and this needs to be tested. Enzicur may be tank-mixed with the fungicides
azoxystrobin, bitertanol, imazalil, potassium bicarbonate and triflumizole and the insecticides abamectin, cyromazin imidacloprid, pirimicarb, pymetrozin, pyridaben, pyriproxifen, spiromesfen, teflubenzuron and thiacloprid. Sulphur is not compatible.

**Biological pesticides**

Enzicur is able to kill bacteria and fungi including entomopathogenic fungal spores and bacteria. Therefore Enzicur should not be tank-mixed with a biopesticide nor sprayed within a few days after a treatment with a bio-insecticide or bio-fungicide since it kills surviving propagules on the leaves. Enzicur once dried has no longer any effect on microorganisms. This means that one day after the usage of Enzicur any biological product can be used without negative effects of Enzicur. Nevertheless, an *in vivo* laboratory test showed that *Bacillus thuringiensis* (Bt) is still active after a treatment of Enzicur. Bt products seem to compatible with the use of Enzicur.

**Conclusion**

A specific formulation and application has been developed with the LP-system for use as an agricultural fungicide-bactericide. Successful control of powdery mildews can be achieved with this formulation. Active substances and product registration are pending in the EU and individual member states. The formulation and its use is patented in the EU and several other countries. The LP-system is a natural system and all the ingredients of the two products are natural chemicals and food approved. The activity is enzyme-based whereby the reactive components are formed in the spray solution, unlike chemical pesticides. Enzicur has a safe profile for animals, plants, and the environment and fits well in IPM systems since it is safe for beneficial arthropods and honeybees and bumblebees. From the mode of action it can be concluded that LPS has a multiple-site activity. Many proteins and enzymes are inactivated in a microorganism and as a result the organism dies. Apart from the oxidation of proteins, other important metabolic processes are disturbed or inhibited. Resistance is very unlikely to develop due to the multiple targeted sites. Moreover, since this is a system present in nature in many organisms as an anti-microbial system, development of resistance is deemed almost impossible. This new product can become an important tool in resistance management programmes.

**References**


BioNem WP: a unique tool for nematode control

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Abstract: Pre-plant application of nematicides is strongly recommended for effective control of nematodes. In certain situations, mainly in perennial crops, or in long season vegetables, nematodes emerge from deep soil layers and thus diminish the effect of the pre-plant treatment. The use of pesticides for post-planting control is limited due to their toxicity to plants and humans. BioNem WP is a biological nematicide, based on a unique strain of Bacillus firmus. It is conveniently applied in commercial fields through irrigation systems. BioNem WP is effective against phytopathogenic nematodes, and is registered in Israel for the control of root knot nematodes in vegetable crops (cucumber, tomatoes, pepper, eggplant and herb crops) and in perennial crops (peaches, olives and ornamentals). Long term suppression of nematode population was observed following single application of BioNem WP, either pre or post-planting. This feature may be attributed to activity in deep soil layers and to persistent nematicidal activity in the treated soil, which was observed even under water recirculation. The long term activity of BioNem WP combined with its safety makes it a unique tool for nematode control in perennials, during harvest of edible crops, and under chronic infestation with nematodes.

Introduction

The effectiveness of methyl bromide in nematode control made the development of new effective nematicides seem unnecessary. As methyl bromide is being phased out, the market is facing the need for an effective nematicide. BioNem WP is a biological nematicide, based on a unique strain of Bacillus firmus. It is effective against phytopathogenic nematodes, and is conveniently applied in commercial fields through irrigation systems. The product is commercially used in Israel for the control of root knot nematodes (Meloidogyne spp., RKN) in vegetables (cucumber, tomatoes, pepper, eggplant and herb crops) and in perennial crops (ornamentals and stone-fruits). Field performance results presented here, combined with its safety and ease of use prove BioNem WP to be an effective and unique tool for nematode control. In these studies, BioNem WP was found especially advantageous for nematode control in perennials, during harvest of edible crops, and under chronic nematode infestation.

Materials and methods

BioNem WP, a wettable powder formulation containing the bacteria B. firmus and additives, Manufactured by Agro-Green, the Biological Div. of Minrav, Ashdod, Israel. All field trials were done in commercial plots. Treated plots were replicated at least 4 times (at least 10 m² per plot) randomly distributed. An aqueous suspension of BioNem WP was injected with a pressure sprayer or fertilizer pump into the irrigation line leading to treated plots. Following application the field received 20 mm of irrigation. To determine soil nematode population, soil from a depth of up to 15 cm was sampled from 8 random locations within the plot. RKN were counted using the Baerman funnel method. Field assessment of nematode damage was carried out with 6-10 plants per plot that were uprooted and the galling index scored on a
severity scale of 0 (clean roots) to 5 (over 75% of the root surface covered with galls). Galling of roots is the measurable damage caused by root-knot nematodes. Damage to the roots is presented as the distribution of galling severity.

Results

Long term suppression of nematodes

Nematode control is best achieved by pre-plant application. When BioNem WP was applied pre-planting in Hypericum (an ornamental crop), reduced nematode population was observed over a period of 10 months. The standard chemical (Cadusafos) provided partial control only, even though it was applied twice (Fig. 1A). The reduced population was reflected also in a greatly reduced damage to the Hypericum roots as assessed 11 months after planting (Fig. 1B). Similar results were seen in pepper, eggplant, and tomato (not shown). Due to the long term effect, only one application was necessary in most annual vegetables.

In perennial crops, the pre-plant treatment cannot provide nematode control which will last the entire life span of the crop. To complement the effect of the pre-plant treatment, annual spring treatments are currently recommended. Treating a peach orchard with BioNem WP in February resulted in some suppression of nematode population (Fig. 2 dotted line). However, earlier application in November that relied on the long term effect of Bionem WP abolished the burst of active nematodes in the following spring (Fig. 2). Such early treatment using conventional nematicides is not considered feasible due to their short term effect.

Figure 1. Long-term suppression of nematodes in hypericum

Figure 2. Effect of application timing on nematode control in peaches
Mid-season application
The effect of pre-plant treatment diminishes with time due to re-infestation of the field. Mid-season application of chemicals is restricted due to their toxic effects, especially during harvesting of edible crops. BioNem WP is non-toxic, non-phytotoxic and is allowed for use during harvesting of edible crops. These safety features coupled with easy application make BioNem WP the preferred candidate for mid-season uses. Application of BioNem WP in Sweet basil (3-months-old) infested with RKN, resulted in a decrease in nematode population while a dramatic increase in nematode population was observed in the untreated plots (Fig. 3A). The benefit of this population reduction was evidenced also by the reduction in the galling of the crop roots. (Fig. 3B). Similar results were obtained in pepper, cucumber, eggplant and tomato (not shown).

![Figure 3. Control of nematodes in Sweet-basil by mid-season application](image)

Persistent nematode control
Soil fumigants eradicate nematodes within hours, after which they vaporize. In contrast, BioNem WP only reduces the nematode population in the soil. Low but measurable numbers of active nematodes can still be found following BioNem WP treatment (1). Despite this incomplete killing, the effect of reduced nematode population lasted for months (Figures 1-3). This phenomenon might be explained by persistent control activity.

Tomato seedlings were planted in soil from BioNem WP treated field that was mixed with freshly infested soil. Roots of these plants had less galling as compared with plants grown similarly in untreated soil (Fig. 4). This effect lasted for 140 days following BioNem WP field application and is the manifestation of persistent nematode control activity in BioNem WP treated soil. None of the chemical nematicides possess this feature, which enable BioNem WP to outperform in certain situations.

Control of chronic infestations
Cropping in detached substrates is supposed to avoid nematode damage. However, once infested, the nematodes spread throughout the plot. Spreading is facilitated by recirculation of water. Chemical control under these conditions is difficult due to leaching. Frequent applications intended to compensate for the short term effect might result in damage to the environment and in reduced efficacy of the pesticides. An example of such situation was found in roses grown on detached substrate (Tuff) and irrigated by water recirculation. These roses were infested with Meloidogyne hapla. Repeated applications of chemical nematicides did not prevent the proliferation of these nematodes. BioNem WP was applied to two rows in the greenhouse. The results in Fig. 5 show an increase in active nematode population in the
untreated rows, while no active nematodes were detected in the BioNem WP treated rows over a period of 12 weeks after application. It is suggested that the persistent nematode control achieved by BioNem WP suppressed nematodes in the treated substrate despite the continuous infestation by the irrigation water.

Conclusions

The results presented here show that BioNem WP provides effective nematode control that favorably compares with current chemical nematicides. Due to the persistent nematode control, BioNem WP was more effective than the chemical nematicides in certain situations. A single pre-plant application of BioNem WP was sufficient for the entire season in annual crops and may be comparable with several chemical nematicide applications per season. A new nematode control regime in perennial crops was demonstrated by autumn application of BioNem WP that enabled nematode control up to and beyond the following spring. A single BioNem WP application controlled nematodes in a plot irrigated by water recirculation. This demonstrates the unique ability of BioNem WP to control chronic infestations. Safety and phyto-safety become a major concern when nematode control is needed during the harvesting of edible crop. BioNem WP provided the needed efficacy along with the required safety.

Reference

Efficacy of some biological agents in controlling pathogenic soilborne fungi infesting watermelon in Egypt

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Abstract: After obtaining preliminary results in vitro, trials were conducted under greenhouse conditions on living plants to determine the antagonistic actions of two antagonistic fungi, *Trichoderma viride*, *Gliocladium virens* and three antagonistic bacteria, *Bacillus subtilis*, *Streptomyces griseoviridis* having commercial name (Mycostop) from Finland, and *Pseudomonas fluorescens* against three fungal soilborne pathogens causing watermelon wilt disease (*Fusarium oxysporum* f. sp. *niveum*, *F. solani* and *Rhizoctonia solani*) through two technical applications, soil drenching and seed coating. The fungicidal and fungistatic effects of some biological fertilizers against the same three pathogenic fungi causing watermelon wilt mentioned before were tested, these were Nitrobien (*Azospirillum* sp. and *Azotobacter* sp.), Phosphorien (*Bacillus megaterium* var. *phosphateum*), Microbien (N-fixing bacteria + phosphorus dissolving bacteria) and Biogien (*Azotobacter* sp.). Two methods were mixing bio-fertilizers with the soil and covering seeds with bio-fertilizers. All treatments gave promising results when compared with controls. Results showed that among the antagonistic fungi *T. viride* proved to be the best, while *S. griseoviridis* (Mycostop®) was relatively more promising than the antagonistic bacteria, while Phosphorien was the best among the tested bio-fertilizers.

Key words: antagonism, biological control, *Citrulius lanatus* L., Plant Growth Promoting Rhizobacteria (PGPR)

Introduction

Watermelon, (*Citrulius lanatus* L.) is one of the most important vegetable cucurbit crops in Egypt and many other countries of the world. In Egypt, the cultivated area was 148262.4 feddans (1 Feddan = 4200 m²) in 2004 growing season yielding approximately 1920000 tons, with average about 12.95 ton/feddan. Egypt has the fifth place in watermelon production globally (FAO Stat. Database, 2005). One of the main restrictions in watermelon and cantaloupe production worldwide is the infestation by different soilborne fungal pathogens, which cause damping off, root rot and wilt diseases. *F. oxysporum* f. sp. *niveum*, *R. solani* and *F. solani* are considered the most vital pathogens which cause the previously mentioned diseases (Cebolla Rosell & Compos Gimeno, 1990; El-Zayat et al., 1993). The first report on watermelon Fusarium wilt in Egypt was published by Melchers (1931) and Fahmy & Menshawy (1932). The infection appears during the growing season at different stages of plant growth from seedling to maturity and causes great losses in yield. Many attempts were carried out for controlling the wilt pathogens infested watermelon including agricultural practices, plant breeding and chemical fungicides applications, however present ecological concerns about fungicide applications are serious and include worker safety, pollution of drainage water and consumer exposure to fungicides residues. Therefore, alternative control
means are highly required (Martyn & Netzer, 1991). The present study aimed to evaluate the antifungal activity of five antagonistic microorganisms, i.e., *T. viride*, *T. harzianum*, *T. humatum*, *G. virens*, *G. roseum* *B. subtilis*, *P. fluorescens* and *S. griseoviridis* against the isolated soilborne fungal pathogens, also to define the fungal suppressive effect of four biofertilizers i.e., Biogien, Microbien, Nitrobien, and Phosphorien, under greenhouse conditions.

**Material and methods**

**Isolation of pathogenic fungal organisms**
Specimens of wilt diseased watermelon plants showing typical symptoms of root rot were collected from different locations of Dakahlia and Damietta governorates at north Egypt. Isolation of pathogenic fungi from roots was done on potato dextrose agar (PDA) using standard isolation techniques.

**Inhibitory effect of certain antagonists on the radial growth of some soil borne fungal pathogens causing watermelon wilt**
The inhibitory effects of five antagonistic fungi, *T. viride*, *T. harzianum*, *T. humatum*, *G. roseum* and *G. virens* isolated from rhizosphere of healthy watermelon plants grown in Dakahlia governorate and antagonistic bacteria *B. subtilis*, *P. fluorescens* and *S. griseoviridis* (commercial product from Finland under commercial name "Mycostop®") on radial growth of *R. solani*, *F. solani*, and *F. oxysporum f. sp. niveum* were studied. Each of obtained fungal antagonist, and the three fungal pathogens were grown on PDA for 5-7 days at (25±2°C), whereas the antagonistic bacteria were grown on Nutrient Agar medium (NA). The untreated control treatment was done on PDA medium surface in Petri dishes by growing one disc of the pathogenic fungus in the same place but there was no antagonist. All plates were incubated at (25±2°C) until control treatment covered the surface of the plate. The inhibition percentage was calculated by comparing the radial growth with the control treatment %.

**Effect of some antagonists on disease incidence in watermelon under greenhouse conditions**
This test was done to evaluate the antagonistic efficacy of the best two fungal antagonists, *T. viride* and *G. virens* - and the three tested bacteria, *B. subtilis*, *P. fluorescens* and *S. griseoviridis* previously tested in lab. against the three tested pathogenic fungi mentioned previously, two application methods, seed treatment and soil drenching were applied; seed treatment was done according to Weller & Cook (1983). Soil drenching application was done according to Bankole & Adebanojo (1996). Control treatments were done by sowing surface sterilized seeds in non-infested soil (as absolute control) and by sowing surface sterilized seeds in infested soil without inoculation with antagonistic fungi (as infested control). The used chemical standard was Topsin M-70 as seed dressing and soil drenching at the recommended doses. The percentage of surviving plants was calculated.

**Effect of some bio-fertilizers on wilt disease incidence of watermelon**
This test was done to determine the ability of four bio-fertilizers; Nitrobien, Phosphorien, Microbien and Biogien on suppressing the diseases incidence and the best method for their application. Tested bio-fertilizers as mentioned before were obtained from The Agriculture Research Center (A.R.C.) in Cairo. Two types of treatments were conducted; seed treatment at the rate of 10 g bio-fertilizer/100 gm seeds then it was sown in the infested soil. Soil treatment was done by mixing the bio-fertilizer with soil at the recommended dose of 50 g/Kg in the upper 5 cm layer according to El-Sheshtawi (2003). The surviving plant percentage was calculated.
Results

Laboratory results showed that all antagonistic fungi affected the radial growth of the tested pathogenic fungi with different grades for example; treatment with *T. viride* reduced the growth of *F. oxysporum* f. sp. *niveum* with 74% when compared with control %, while the antagonist *G. virens* reduced radial growth of *F. solani* and *R. solani* with 72 and 61%, respectively. Among all antagonistic bacteria, *S. griseoviridis* reduced the radial growth of *F. oxysporum* f. sp. *niveum* with 50% comparing with control %, while *B. subtilis* reduced the radial growth of *R. solani* with 82% while, *P. fluorescens* reduced growth of *R. solani* with 62%. Under greenhouse, results showed that the antagonistic effect of *T. viride* gave better results than *G. virens*. *T. viride* gave 80% living plants, where *G. virens* gave 64%. In the same experiment, the chemical control standard (Topsin M-70) gave 82% living plants when applied as seed coating. The untreated control gave 49% living plants. Result proved that the soil drenching technique gave a better living plants ratio than the seed coating treatment.

![Figure 1](image1.png)

**Fig. 1.** Effect of application with some antagonists as soil drenching on watermelon wilt disease incidence caused by some soilborne fungi

Results obtained from bacteria could be resumed as follows: Mycostop (*S. griseoviridis*) gave 77% living plants where the untreated control gave 49% when the chemical standard Topsin M-70 gave 86% living plants, followed by *B. subtilis* with 71% then *P. fluorescens* with 61% living plants. Results showed no significant difference between soil drenching and seed coating methods (Figs 1 and 2). Results obtained from bio-fertilizers under greenhouse conditions showed that mixing bio-fertilizers with soil gave better results than covering seeds
with bio-fertilizers. The most effective fertilizer was Phosphorien giving 73% living plants followed by Biogien at 65%. The untreated control gave 49% living plants, when the chemical fungicidal control Topsin m-70 gave 86% living plants (Figs 3 and 4).

![Fig. 2. Effect of application with some antagonists as seed dressing on watermelon wilt disease incidence caused by some soilborne fungi](image)

**Discussion**

Biological control of soil borne fungi on various crops has much potential for disease management, though there are several problems with practical implementation (Cook & Baker, 1983; Lewis & Larkin, 1998). Numerous organisms demonstrated biocontrol capability against soil borne fungi in the present study, but there was also much variability in control from one trial to the next. From results obtained, we resume the discussion of the best antagonistic efficacy as follows: a) antagonistic fungi: - *T. viride* proved to be the best among the tested fungal antagonists according to the results of parameter of antagonistic action and living plants ratio. This result may be explained by the high capability of the exudation of lytic enzymes including chitinase, β-1,3 glucanase, β-glycosidase and protease which are thought to be involved in mycoparasitism. Also *T. viride* has a strong competitive ability for consuming carbon sources (Antal et al., 2000). b) antagonistic bacteria: - among all tested antagonistic bacteria, we noticed that the best results were obtained with *S. griseoviridis*, its antifungal activity probably related to some antibiotics such as quinolone and coumorin (novobiocin). In addition, the bacterium may deprive the pathogen of living space and nourishment by colonizing the plant roots in advance of the pathogen (Gregory et al., 2001). c) bio-fertilizers: - Among all tested bio-fertilizers, it was visible noticed that Phosphorien was the most effective in suppressing wilt disease incidence. This effect of Phosphorien can results from one or more mechanisms including biological control by competition, production
of siderophores or antibiotics, induced disease resistance, direct growth promotion through phytohormone production and increased nutrient availability through nitrogen fixation and solubilization of organic and inorganic phosphate (Mujeebur & Khan, 2001). In addition, phosphorus itself play a vital role in building the natural defense of plants against pathogens (Francê Ois, 1984).

Fig. 3. Effect of application with some bio-fertilizers mixing with soil on watermelon wilt disease incidence caused by some soilborne fungi

Fig. 4. Effect of application with some bio-fertilizers as seed coating on watermelon wilt disease incidence caused by some soilborne fungi
References


First report of biocontrol activity of *Pseudomonas reactans*, pathogen of cultivated mushrooms, against strawberry powdery mildew in greenhouse trials

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**Abstract:** Powdery mildews are diseases serious damaging several crops. As most are ecto-parasites they are a favoured test target to potential biocontrol agents. *Pseudomonas reactans*, a fluorescent pseudomonad responsible, besides other bacteria including *P. tolaasii*, for the brown blotch disease of *Agaricus bisporus* and yellowing of *Pleurotus ostreatus* and *P. eryngi*, produces the lipodepsipeptide (LDP), WLIP (White Line Inducing Principle). The aim of this study was to evaluate *P. reactans* USB20 efficacy in powder mildews biocontrol. Two systems were selected: *Podosphaera aphanis*/strawberry and *Erysiphe necator*/grapevine. *Ps. reactans* broth with cells, broth culture without cells and cells in water suspension were directly sprayed on strawberry leaves prior inoculated with *Podosphaera aphanis* under controlled greenhouse conditions. *Ps. reactans* whole culture was also applied on grape leaves prior inoculated with *Erysiphe necator*. Disease severity and incidence were assessed. Both the whole culture and broth culture of *Ps. reactans* inhibited strawberry powdery mildew development, but the former was ineffective against grape powdery mildew. *Ps. reactans* metabolites, including WLIP, appear to have a toxic effect on *P. aphanis* and not on *E. necator*, which let us inferring that they have a differential spectrum of activity against powdery mildews.

**Key words:** brown blotch, grapevine, lipodepsipeptides, tolaasiins, White Line Inducing Principle

**Introduction**

Powdery mildews are serious diseases of several cultivated crops. Powdery mildews are primarily controlled by chemical fungicides, like sulphur, strobilurins and triazoles which, however, have some limitation since sulphur is toxic to beneficial and predator mites and has a phytotoxic effect on the plants; strobilurins and triazoles induce the development of pathogen resistance and triazoles leave residues on fruits. Furthermore, the lack of good resistant cultivars and the increasing request for quality and food safety have fostered research efforts to develop control measures alternatives to chemicals, as plant extracts, oils, salts, and biocontrol agents. Up to now several biocontrol agents have been tested against powdery mildews, such as *Ampelomyces quisqualis*, a fungus able to parasite powdery mildews and commercially available as AQ10 (Daoust & Hofstein, 1996), but no experiments were made to use bacteria of the genus *Pseudomonas* as biocontrol agents. *Ps. tolaasii* and *Ps. reactans* are fluorescent pseudomonads responsible for the brown blotch disease of *Agaricus bisporus* and yellowing of *Pleurotus ostreatus* and *P. eryngi* (Iacobellis & Lo Cantore, 2003) and
produce two lipodepsipeptides (LDPs), peptidic secondary metabolites, called tolaasiins and WLIP (White Line Inducing Principle), respectively. Some studies have indicated tolaasiins as the main virulence factor of *Ps. tolaasii* toward *A. bisporus* (Rainey et al., 1992; Soles-Rivas et al., 1999). The brown blotch symptoms are the result of the direct action of the tolaasiin on the *A. bisporus* cytoplasmic membranes, which represent the principal target of the toxin. Furthermore, tolaasiins inhibit microbial growth and, in particular, of phytopathogenic fungi. The biological properties of WLIP are less known, although recent investigations have pointed out its ability to alter tissue blocklets of *A. bisporus*, to inhibit the growth of different fungi and bacteria, to lyse erythrocytes through transmembrane pore formation (Lo Cantore et al., 2003; Lo Cantore et al., 2006). Considering the above features it seemed of interest to evaluate the biocontrol ability of *Ps. reactans* against some powder mildew agent. Here we report the preliminary results.

**Material and methods**

The strain USB20 of *Ps. reactans* isolated from cultivated mushrooms was tested in two pathogen/host systems: *Podosphaera aphanis*/strawberry and *Erysiphe necator*/grapevine under greenhouse controlled conditions. Treatments, including standards (used at commercial dosages) and untreated control were arranged in fully randomised blocks (Table 1).

**Podosphaera aphanis*/strawberry system**

Four replicates per treatment were used; replicate size was of three potted plants. *P. reactans* USB20 was grown in nutrient broth for 24 h and then directly sprayed on strawberry leaves either as whole culture (cells plus broth), as washed cells in a water suspension and as broth without cells. Artificial inoculation was done shaking mildew infected leaves on the treated plants. Treatment applications were repeated weekly for 5 times. Disease severity (percent of diseased leaf area) and incidence (percent of leaves with disease symptoms) were assessed weekly, scoring 9 randomly chosen leaves per replicate, and AUDPC (Area Under Disease Progressive Curve) was calculated with all collected data on disease severity from first application until the 4th week. Data were analyzed by analysis of variance ANOVA and means were compared using the HSD test at \(P \leq 0.05\).

**Erysiphe necator*/grapevine system**

Grapevine plants were treated as described for strawberry but only with the whole culture. Inoculation was performed as for strawberry mildew, five replicates of one plant each. Disease severity and incidence were assessed on all leaves at the end of the experiment. Significant differences among treatments were determined by HSD test at \(P \leq 0.05\).

**Table 1. Treatments used in the two selected systems**

<table>
<thead>
<tr>
<th><em>Podosphaera aphanis</em>/strawberry</th>
<th><em>Erysiphe necator</em>/grapevine</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ps. reactans</em> USB20 whole culture</td>
<td><em>Ps. reactans</em> USB20 whole culture</td>
</tr>
<tr>
<td><em>Ps. reactans</em> USB20 cells in water</td>
<td>Sulphur (Thiovit, Syngenta)</td>
</tr>
<tr>
<td><em>Ps. reactans</em> USB20 broth culture</td>
<td><em>Ampelomyces quisqualis</em> (AQ10, Intrachem Bio)</td>
</tr>
<tr>
<td>Sulphur (Thiovit, Syngenta)</td>
<td>Untreated control (water)</td>
</tr>
<tr>
<td>Untreated control (water)</td>
<td></td>
</tr>
</tbody>
</table>
Results and discussion

Applications of *Ps. reactans* USB20 on strawberry plants led to a reduction in powdery mildew when compared to the untreated control. *Ps. reactans* whole culture and broth culture were the most effective. *Ps. reactans* cells alone did not have any significant effect (Fig. 1).

![Graph showing severity of strawberry powdery mildew](image)

**Figure 1.** Severity of strawberry powdery mildew in greenhouse through five weekly applications of *Ps. reactans* USB20 culture.

Disease was evaluated weekly from the first application until the 4th week and AUDPC (Area Under Disease Progressive Curve) was calculated. Means followed by the same letter do not differ to each other according to HSD test at $P \leq 0.05$.

Grapevine powdery mildew (*E. necator*) was not inhibited by treatments with the broth plus cells of *Ps. reactans* USB20 (Table 2). Only sulphur, the chemical standard, significantly reduced the incidence and severity of the disease. The absence of a toxic effect of the whole culture on *E. necator* contrasts with the result on *P. aphanis*. The above results suggest that *Ps. reactans* metabolites, including WLIP, may be important in the observed biocontrol effect. The lack of antagonistic effect against *E. necator* may be due to a different sensitivity of the latter to *Ps. reactans* metabolites.

Table 2. Effect of five weekly applications of *Pseudomonas reactans* USB20 whole culture on grape powdery mildew in greenhouse conditions. Data show the disease incidence and severity evaluated two weeks after the last treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence (%)</th>
<th>Severity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control (water)</td>
<td>100.0 a</td>
<td>13.5 ab</td>
</tr>
<tr>
<td>Sulphur</td>
<td>44.0 b</td>
<td>2.3 c</td>
</tr>
<tr>
<td><em>A. quisqualis</em></td>
<td>94.4 a</td>
<td>18.8 b</td>
</tr>
<tr>
<td>USB20 whole culture</td>
<td>96.7 a</td>
<td>9.8 a</td>
</tr>
</tbody>
</table>

Means followed by the same letter do not differ to each other according to HSD test at $P \leq 0.05$. 
The potential ability of \emph{P. reactans} as biological control agent has been recently reported. In fact, Faltin et al. (2004) showed the ability of some \emph{Pseudomonas} spp. strains, resembling \emph{Ps. reactans} and obtained by other niches different from mushrooms, to control \emph{Rhizoctonia solani} in sugar beet.

Acknowledgements

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Evaluation of new biological control agents against grapevine powdery mildew under greenhouse conditions

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Abstract: Pathogen resistance to fungicides, concerns regarding pesticide residues and revocation of some widely used fungicides have increased research efforts for developing biocontrol agents of fungal pathogens. Natural fungicides active against grapevine powdery mildew are few and moderately effective. Moreover, no good commercial powdery mildew-resistant grapevine varieties are available. Therefore, development of alternatives to chemical pesticides against Erysiphe necator is of high priority. Efficacy trials were carried out during 2004 and 2005 using bacteria, yeasts, fungi, plant extracts and Electrolyzed Acid Water (EAW) to control powdery mildew under controlled greenhouse conditions. Only few of the tested yeasts, bacteria and fungi were significantly effective in reducing powdery mildew symptoms. Among natural products, only an enzyme based fungicide (KBV 99-01), when sprayed three days after powdery mildew inoculation, was effective. Promising results were obtained with daily sprays of EAW during one week after inoculation and EAW applied in alternation with a yeast suspension.

Key words: Erysiphe necator, microorganisms, natural substances, plant extracts

Introduction

Fungicide resistance problems, concerns regarding pesticide residues and revocation of some widely used fungicides have increased research efforts for developing biocontrol agents (BCAs) of fungal pathogens. In addition, the lack of good resistant cultivars has increased the interest in alternative methods to control of powdery mildews (Kiss et al., 2004). The efficacy of alternatives to chemical fungicides in powdery mildew control, such as oils, salts, soluble silicon and plant extracts, have widely been studied, especially in greenhouse production (Belanger & Benyagoub, 1997; Pasini et al., 1997; McGrath & Shishkoff, 1999). Many potential BCAs have also been tested, but only few of them showed a good disease control under field conditions. The aim of the present study was to evaluate the efficacy of microorganisms and some new compounds against grapevine powdery mildew under controlled greenhouse conditions, as a first step in the process of selecting new alternatives to chemical fungicides.

Material and methods

The inhibition efficacy of Erysiphe necator by a single application 6 hours before artificial inoculation of several fungi, bacteria and yeasts isolated from natural environment was preliminary evaluated (Table 1). Sulphur, water and three commercial biocontrol preparations
(TRICHODEX, Clonotri and Subic) were used as standards. The microorganisms were isolated in Israel (Y16, Y13, B69, B6), and in Italy in Apulia and Trentino Region (Pseudomonas tolasii USB 1, P. reactans USB 20, Bacillus subtilis F77, Alternaria alternata, 18 bacteria, 23 yeasts and 9 fungi named here with different codes).

In the preliminary trials the experimental microorganisms were grown in Petri dishes on PDA at 25°C for 48 hours, suspended in distilled water containing 0.1% Tween 80 and sprayed on plants until complete wetting under greenhouse conditions. The main trial was split into four groups. The most effective microorganisms in the preliminary trial, Electrolyzed Acid Water (EAW) and an enzyme based fungicide (Table 2) were tested against grapevine powdery mildew in two greenhouse trials. Sulphur (Thiovit, Syngenta) was used at 3 g/l as standard control; distilled water containing 0.1% Tween 80 was used as untreated control in all the experiments.

Table 1. Microorganisms, standards (sulphur and three commercial BCAs) and untreated control (water) tested in the preliminary greenhouse trial

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Commercial name or code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphur</td>
<td>Thiovit</td>
</tr>
<tr>
<td>Distillate water +0.1 % Tween 80</td>
<td>Untreated control</td>
</tr>
<tr>
<td>Trichoderma harzianum T39</td>
<td>TRICHODEX</td>
</tr>
<tr>
<td>Clonostachys rosea + T. harzianum</td>
<td>Clonotri</td>
</tr>
<tr>
<td>Bacillus licheniformis + B. subtilis</td>
<td>Sublic</td>
</tr>
<tr>
<td>T. konigii</td>
<td>cl1</td>
</tr>
<tr>
<td>Beauveria bassiana</td>
<td>cl2, cl14, cl5</td>
</tr>
<tr>
<td>Trichoderma spp.</td>
<td>cl6</td>
</tr>
<tr>
<td>T. atroviride 122F</td>
<td></td>
</tr>
<tr>
<td>Fungi (experimental)</td>
<td></td>
</tr>
<tr>
<td>Yeasts (experimental)</td>
<td></td>
</tr>
</tbody>
</table>

Treatments were applied 6 hours before inoculation.

In the first greenhouse trial the tested microorganisms were grown in a liquid culture (NB or YMDB) at 25°C for 48 hours, centrifuged and re-suspended as in the preliminary trial. The two isolates of P. reactans (USB 1 and USB 20) and Alternaria alternata were sprayed with their culture broth, since in previous experiments it was demonstrated that they produce toxic metabolites. In the second greenhouse trial EAW, EAW+Y13, Y13 and the enzyme based fungicide KBV 99-01 were compared with Sulphur and untreated control.

All the suspensions were adjusted to 1×10^7 cells/ml for fungi and 1×10^8 CFU/ml for bacteria and yeasts. For each treatment, 100 ml of suspension were sprayed. Five replicates (one plant each) of the cultivar Pinot Gris having one shoot with 5-6 green and fully expanded leaves were used. Plants were grown under controlled conditions (25°C and 60% R.H.) in a pathogen free greenhouse. Powdery mildew inoculation was done by shaking above the plants infected leaves with fresh and heavy sporulating lesions collected in the field. Assessments were done 7, 14, 21 and 28 days after inoculation. The percentage of infected leaf area (severity) and the
percentage of infected leaves (incidence) were assessed on all leaves. Analysis of variance (ANOVA) was applied on “Arcsin” transformed data, using the software Statistica 7 (Statsoft, Italy). Significant differences between treatments were determined by Duncan’s test. The assessment with the highest level of disease on the untreated control is presented in the results.

Table 2. Control agents tested in the first and second greenhouse trials

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Name of control agent</th>
<th>Application time vs. inoculation(I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphur</td>
<td>Thiovit</td>
<td>6 hours before I</td>
</tr>
<tr>
<td>Distilled water+Tween 80</td>
<td>Water</td>
<td>daily</td>
</tr>
<tr>
<td>Enzyme based fungicide</td>
<td>KBV 99-01</td>
<td>3 days after I</td>
</tr>
<tr>
<td>Electrolyzed Acid Water</td>
<td>EAW (CBCE)</td>
<td>daily</td>
</tr>
<tr>
<td>Electrolyzed Acid Water+yeast</td>
<td>EAW (CBCE)+Y13</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td><em>Alternaria alternata</em></td>
<td>-</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td>Fungus (experimental)</td>
<td>fbsa</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td>Yeast (experimental)</td>
<td>yssa, ycsb, ysmmb, yrsb, ybps</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td>Yeast (experimental)</td>
<td>Y13</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td>Yeast (experimental)</td>
<td>Y16</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td>Bacterium (experimental)</td>
<td>bbnm1</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td>Bacterium (experimental)</td>
<td>B69</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td>Bacillus <em>subtilis</em></td>
<td>F77</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td><em>Pseudomonas tolasii</em></td>
<td>USB 1</td>
<td>6h before, 3, 7 days after I</td>
</tr>
<tr>
<td><em>Pseudomonas reactans</em></td>
<td>USB 20</td>
<td>6h before, 3, 7 days after I</td>
</tr>
</tbody>
</table>

**Results and discussion**

During the trials, disease pressure was high; disease incidence was used to compare the efficacy of tested agents. In the first group of microorganisms of the preliminary greenhouse trial, three fungi and six yeasts were able to reduce disease incidence as sulphur. The best results were obtained using Y13, a yeast isolated in Israel (Fig. 1). In the second group two yeasts (ysma and yccb) and two bacteria (bbnm2 and bbpm2) were as good as sulphur. The fungus cl5 and a yeast isolated in Trentino (ygsc) reduced the disease incidence, though to a lesser extent compared to sulphur (Fig. 2). In the third and fourth groups 5 fungi (CL2, CL6, flma, fcca, fmma), 11 bacteria (B69, B71, bmrb, bgma, bgsb, bsmmb, bssb, bbn, bbns1, bbnm1, bbpm1) and 11 yeasts (ylsa, ylcmb, yrscb, ysmmb, yrsb, yssc, ypsc, ypsw, ybps, ypsa, ybnm) were compared, but no significant difference were obtained with sulphur (data not shown).

In the first greenhouse trial three bacteria (B69, B6 and bbnm1) and four yeasts (yssa, ycsb, yrsb and ybps) were effective compared to sulphur, whilst all other agents only partially reduced powdery mildew infections compared with the untreated control (water). The best results were obtained using the yeast yrsb (Fig. 3).

In the second greenhouse trial EAW sprayed in combination with the yeast Y13 seems to be an effective ingredient for powdery mildew control. The enzyme based fungicide (KBV 99-01) sprayed on the third day after inoculation showed as good results as sulphur in powdery mildew control (Fig. 4). Microorganisms grown and sprayed with their culture broth (USB 1,
USB 20 and *Alternaria alternata* showed high phytotoxicity on leaves, therefore were not included in the analysis.

Figure 1. Powdery mildew incidence on plants treated with of fungi, yeasts and bacteria sprayed 6 hours before inoculation in the preliminary greenhouse trial (first group), 28 days after inoculation. Columns with same letters do not significantly differ ($P<0.05$, Duncan's test).

Figure 2. Powdery mildew incidence on plants treated with of fungi, yeasts and bacteria sprayed 6 hours before inoculation in the preliminary greenhouse trial (second group), 28 days after inoculation. Columns with same letters do not significantly differ ($P<0.05$, Duncan's test).
Several non-chemical alternatives gave interesting results against grapevine powdery mildew. Some of the microbial control agents gave promising results in reducing the disease under controlled conditions. They will be further studied in greenhouse experiments to evaluate their mechanism of action, best time of application, survival etc. in order to develop alternatives to sulphur against powdery mildew in organic viticulture. For the field experiments the selected organisms must be formulated to reduce their variability in disease
control and increase their survival and efficacy. Furthermore, the integrated use of EAW with biocontrol agents seems to be helpful in increasing disease suppression. Another good alternative to sulphur could be the enzyme based fungicide (KBV 99-01), but the positive results should be confirmed.

Acknowledgements

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References

Potential new applications of Shemer, a *Metschnikowia fructicola* based product, in post-harvest soft fruit rots control

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Abstract: Currently, the commercial formulation Shemer of a selected strain of *Metschnikowia fructicola*, is successfully applied to prevent the development of post-harvest rots caused by a wide range of phytopathogenic fungi, including *Aspergillus*, *Botrytis*, *Penicillium* and *Rhizopus*. The formulated product is currently registered in Israel for use on grapes, strawberries, citrus and sweet potatoes. Soft fruits are economically important crops for Italian agriculture; but they are at risk to be infected by rotting fungi resulting in high yield losses. Moreover, no fungicide against post-harvest rots of soft fruits is currently permitted as fruits for fresh consumption have zero, or at least below efficacious level, as maximum residue level. Hence there is a high interest and need to develop new control tools for post-harvest diseases. The aim of this work was to investigate the control efficacy of Shemer® against post harvest rots caused by *B. cinerea*, *P. expansum*, *R. stolonifer* and *Monilia fructigena* on several soft fruits. Small scale post-harvest treatment was carried on strawberry, blueberry, red currant, raspberry, peach and nectarine artificially inoculated with each of the four mentioned fungi. Field application of Shemer on soft fruits just before harvest was mainly target to control natural *B. cinerea* infection. Initial results suggest that post-harvest rot control efficacy of Shemer® is strongly dependent of the combination of fruit type, pathogen and storage temperature.

Key words: efficacy, post-harvest rot, yeast

Introduction

Post-harvest fungal diseases, resulting in major losses of fruits and vegetables, can be successfully controlled by chemical fungicide. Development of fungicide resistance in pathogens and increasing concerns on fungicidal residues has stimulated the search for alternative measures for disease control (Yao & Tian, 2005). Yeasts have been focused on as antagonists of post-harvest pathogens because they are naturally occurring on fruits and can colonize fruit skin and wounds, thus preventing the establishment of rotting agents. The ascosporic yeast *Metschnikowia fructicola*, was identified as a good biocontrol agent against post-harvest fruit rot (Kurtzman & Droby, 2001). It was already tested with promising results on strawberry (Karabulut et al., 2004) and table grapes (Karabulut et al., 2003).

Soft fruits are economically important crops for Italian agriculture; but they are readily attacked by *Botrytis cinerea* and other fungal genera such as *Penicillium*, *Rhizopus* and *Monilinia* resulting in high yield losses. Moreover, no fungicide against post-harvest rots of soft fruits is currently permitted, as fruits for fresh consumption have zero, or at least below efficacious level, maximum residue level. Hence there is high interest and need to develop new post-harvest diseases control tools. The aim of this work was to identify new potential application of *M. fructigena* using four fungal pathogens on six different soft fruits.
Material and methods

Experiments were carried out during summer 2005 in Trentino. In small scale post-harvest treatments *M. fructicola* was applied on fruits immediately after harvest. Red currant (cv. Rovada), high-bush blueberry (cv. Brigitta), raspberry (cv. Tulameen), strawberry (cv. Elsanta), nectarine and peach were used. Fruits were treated by dipping in 0.2% suspension of Shemer® (Agrogreen, Israel), a commercial product containing at least $10^{10}$ cells/g of *M. fructicola*. Iprodione (Rovral, Bayer) at the concentration of 0.3% and water were used as chemical standard and untreated control, respectively. Four wounds were made before treatment at the equator of peach and nectarine using a sterile needle. Different set of fruits were inoculated spaying suspensions of each of the following pathogens: *B. cinerea*, *M. fructigena*, *R. stolonifer* and *P. expansum* ($5 \times 10^4$ conidia/ml) isolated from decayed fruits. Three peaches and three nectarines (replicates) were used for each combination of fruit, pathogen, treatment and temperature; assays on berries were carried out on four plastic box (replicates) containing respectively 5 strawberry, 10 blueberry, 30 redcurrant and 8 raspberry fruits.

Field trials were carried out in Valsugana on ripen red currant, blueberry and raspberry of the same varieties of post-harvest experiments. No treatment was earlier applied in the orchards. Plants were treated twice (one week and two hours before harvest). Before harvest fruits were artificially inoculated by spraying a suspension of *B. cinerea* ($10^5$ conidia/ml, 0.2 litre/plant). Ten plastic boxes (replicates) containing 8 raspberries, 30 blueberries or 80 redcurrant (4 bunches) were used in the assessments for each temperature of storage.

In both post-harvest and pre-harvest trials fruits were stored for 10 days at 1°C and thereafter transferred at 25°C for disease evaluation or were constantly kept at 25°C. Disease on peach and nectarine was assessed as severity (percentage of diseased fruit area); disease on soft fruits and strawberry was assessed as incidence (percentage of berries with symptoms). Fruit observation started 24 hours after transferring fruits at 25°C until disease severity or incidence reached 70-80%. Assessments showing the highest diseases incidence or severity on the untreated control where statistically analysed. ANOVA on arcsin transformed data was used; Duncan’s Multiple Range Test was applied to separate means for post-harvest trial, and T-test was used for field trials.

Results

With post-harvest treatments Shemer significantly reduced rots on strawberry at both temperatures as good as the chemical standard, except for strawberry inoculated with *R. stolonifer* stored at low temperature where there was a reduction compared to the untreated control but to a lesser extend compared to the chemical (Table 1). Rot symptoms reduction achieved by Shemer was significant also on raspberry after storage at 1°C for all the pathogens; at 25°C acetic bacteria are quickly attacking the fruits, making it impossible for evaluation, except for *B. cinerea* that was significantly reduced compared to the untreated but not as with the chemical. Shemer was not able to control rots on red currant, apart from *P. expansum* at 1°C. *M. fructigena* was reduced but not significantly compared to the chemical on blueberry, peach and nectarine at both temperatures. Shemer was not effective against *B. cinerea* on blueberry at 1°C and partially effective in the other combinations of pathogen/temperature. *R. stolonifer* did not develop on blueberry. On peach and nectarines the results were variable depending on the specific combination of fruit, pathogen and temperature (Table 1).
Table 1. Results of small scale post-harvest trial with artificial inoculation of pathogens on fruits

<table>
<thead>
<tr>
<th></th>
<th>Monilia fructigena</th>
<th>Botrytis cinerea</th>
<th>Penicillium expansum</th>
<th>Rhizopus stolonifer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1°C 25°C</td>
<td>1°C 25°C</td>
<td>1°C 25°C</td>
<td>1°C 25°C</td>
</tr>
<tr>
<td>Strawberry</td>
<td>+ + + + + ± +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raspberry</td>
<td>+ nd + ± + nd + nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Redcurrant</td>
<td>- - - + - - -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blueberry</td>
<td>± ± - ± ± nd nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peach</td>
<td>± ± - + ± ± + -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nectarine</td>
<td>± ± + - - - ±</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fruits were incubated at 1°C (10 days) and 25°C. Incidence of rotten fruits was assessed, except for peach and nectarine, where severity was used. “+”: significant control by Shemer and as good as chemical standard; “±”: significant control by Shemer, but lower than chemical standard; “-”: no differences in the use of Shemer compared to the untreated control; “nd”: not determined (acetic bacteria infections)

Pre-harvest application on red currant, after twenty days of incubation at 25°C, showed significant differences between treated and untreated fruits (Table 2). Grey mould developed quickly after transferring berries from low temperature (1°C) to room temperature (25°C): after ten days average incidence on berries was 32% on treated samples and significantly different from the 53% on untreated ones. *B. cinerea* was not significantly controlled by Shemer on blueberry. Efficacy of Shemer was high on raspberry (Table 2).

Table 2. Results of fruit rots incidence, pre-harvest treated with Shemer® (*M. fructicola*)

<table>
<thead>
<tr>
<th></th>
<th>Botrytis cinerea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1°C 25°C</td>
</tr>
<tr>
<td>Redcurrant</td>
<td>Shemer   32 a 18 a</td>
</tr>
<tr>
<td></td>
<td>Untreated 53 b 39 b</td>
</tr>
<tr>
<td>Blueberry</td>
<td>Shemer   21 a 33 a</td>
</tr>
<tr>
<td></td>
<td>Untreated 45 a 37 a</td>
</tr>
<tr>
<td>Raspberry</td>
<td>Shemer   35 a 9 a</td>
</tr>
<tr>
<td></td>
<td>Untreated 90 b 56 b</td>
</tr>
</tbody>
</table>

*B. cinerea* was artificially inoculated and fruits were incubated at 1°C (10 days) and 25°C. Data in the same column for each type of fruit, followed by the same letter are not significantly different according to Duncan Test ($P<0.05$).

Discussion

The efficacy of Shemer in post-harvest treatment (Table 1) seems to be more dependent on the fruit type than on temperature or pathogen. In particular Shemer is highly active against rots on strawberry and raspberry, the effect is almost absent on red currant. The high control
efficacy of *B. cinerea* on raspberry and its partial effect con Blueberry was also confirmed by Shemer pre-harvest treatment. Pre-harvest application of Shemer on red currant resulted in a good rot control, which differs from post-harvest trials. The higher *B. cinerea* control on red currant obtained by pre-harvest than by post-harvest treatment is probably related to a better fruit skin colonization of *M. fructicola*. In fact two application of Shemer (one week and two hours before harvest) where applied in pre-harvest trial and only one treatment just 6 hours before pathogen inoculation was applied in post-harvest trial.

Rot development was faster on fruit kept in cold storage than in fruit constantly kept at room temperature and Shemer was in general less active in cold stored fruits. This phenomenon is probably due to *B. cinerea* ability to grow even at low temperature, which gives to the pathogen a competitive advantage versus the antagonistic yeast.

These preliminary results suggest that Shemer can be successfully applied on new crops, although additional tests under commercial conditions are necessary to confirm the data obtained and to identify the most effective mode and time of application for each crop.

**Acknowledgments**

We thank F. Bampi for technical help and C. Longa for pathogen identification during the assessments. Shemer was kindly provided by Agrogreen, Israel. This research was supported by SafeCrop Centre, funded by Fondo per la Ricerca, Autonomous Province of Trento.

**References**


Potential of *Lentinula edodes*, *Agaricus blazei* and *Saccharomyces cerevisiae* in the control of *Guignardia citricarpa*, the causal agent of post-harvest citrus black spot

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**Abstract:** Brazil is considered the biggest citrus producer and the biggest orange juice exporter. Post-harvest diseases represent a great loss in the citriculture, and for many fruits to be exported they should be free of chemical residues. In relation to some pathogens in post-harvest, it can be mentioned *Guignardia citricarpa*, the causal agent of citrus black spot, that has a great economic importance by interfering in production and causing aesthetic depreciation of the fruits that can reduce commercialization of fresh-fruits in the external market. Because of the economical importance of this disease, and the control difficulties, the search for alternative control measures that can make possible improve the producing capacity of the growers and the obtaining of fruits with excellent quality are indispensable.

We studied the viability of using *Lentinula edodes* (Shiitake mushroom), *Agaricus blazei* (medicinal mushroom) and *Saccharomyces cerevisiae* (yeast strains used in fermentative processes) as biocontrol agents of citrus black spot control. The first part of the study involved the possible biological control and resistance induction in the fruits of *Citrus sinensis* var. Valência by the fungi. It was possible to observe that the aqueous extracts from *L. edodes* and *A. blazei* basidiocarps reduced the formation of new lesions caused by *G. citricarpa* in the sweet orange fruits. In the second part of the study, the controlling potential of *S. cerevisiae* against *G. citricarpa* was checked in vitro. By using plate assays, it was shown that among the tested strains (BG-1, CR-1, CAT-1, KD-1, K-1 and PE-2), the yeast strain CR-1 was the one that exhibited the greatest antagonist activity against the phytopathogen by reducing mycelium growth. It was also demonstrated that the strains were able to produce volatile compounds with fungistatic action inhibiting up to 83% the development of the pathogen. Thus, it was possible to show that the mushrooms and the strains of *S. cerevisiae*, especially strain CR-1, are potential agents for the control of *G. citricarpa*. And in the case of the yeast, one of the inhibition mechanisms involves the production of volatile compounds.

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Selection of crude fungal extracts with potential of control of Botrytis cinerea in tomato (Lycopersicon esculentum Mill.)

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2 Pontificial Javeriana University

Abstract: A preliminary screening of fungal secondary metabolites produced in Czapeck-Dox and YMG culture media with potential applications for Botrytis cinerea control was conducted by evaluating the inhibitory effect of 172 crude extracts from 86 isolates of filamentous fungi previously isolated from three different agroecological sites of the Amazonian region of Colombia. When turbidimetric technique was used, a calibration model explaining 64% of the conidial germination of B. cinerea was obtained. Twenty two extracts (15 produced on YMG medium and 7 on Czapeck-Dox medium) inhibited conidial germination of B. cinerea more than 60% as compared with the untreated pathogen control. Four extracts inhibited B. cinerea germination between 85 and 90% after 16 h of incubation: two of these extracts were produced on YMG with Penicillium sp. strain 44 and Beauveria sp. strain 223, the two others were produced on Czapeck-Dox with Aspergillus sp. strain 110 and Phialophora sp. strain 150. When the biocontrol activity of these four extracts was evaluated against B. cinerea, two of them (Penicillium sp. and Aspergillus sp.) showed phytotoxic activity in tomato leaflets, the obtained from Phialophora did not reduce disease severity while extract from Beauveria sp. reduced disease severity in 74%. Three evaluation criteria were considered, first, the determination of the effect of crude extracts over B. cinerea growth by using a turbidimetric technique on 96-well microtitre plates, second, the microscopic analysis of the fungus germination, and third, a bioassay on detached leaves to determine the biocontrol effect produced by the crude extracts.

Key words: antifungal activity, crude fungal extract, 96-well microtitre assay

Introduction

Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth and filamentous fungi isolated from these ecosystems represents a rich source of secondary metabolites and bioactive compounds with potential use in medicine, agriculture, and industry (Strobel & Daisy, 2003). The discovery of several substances from crude fungal extracts could provide high and sometimes specific activity against developmental stages involved in pathogenesis of plant-pathogenic fungi, such as conidial germination and can be used as products per se or like “lead molecules” for the development of new agricultural fungicides if their mode of action is sufficiently selective (Gullino et al., 2000; Thines et al., 2004). These substances could represent alternatives to control epidemic outbreaks produced by Botrytis cinerea which causes grey mould on a wide range of crops (Meyer et al., 2001) during pre- and postharvest (Malolepsza, 2004), causing heavy damages and losses. The objective of this investigation was to select extracellular fungal extracts inhibitory to B. cinerea in vitro germination and efficient to control disease in planta.
**Materials and methods**

**Microorganism and growth conditions**

Eighty-six isolates of unidentified filamentous fungi originally isolated from northwest Leticia, Amazonas (Colombia) by Cabrera (2000) growing on Sabouraud agar (pH 4.5) during 8 days at 26±2°C were used. To produce the extracellular crude extracts two liquid media were tested: 25 ml of Czapeck-Dox (CD) or yeast malt glucose (YMG) medium (pH 7.0 before sterilization) containing Tween 80 0.1% in 125 ml flasks were inoculated with 1×10⁶ conidia/ml or five agar plugs (6 mm diameter for sterile mycelia fungi) and incubated on a rotatory shaker at 160 rpm at 26±2°C for 7 days.

*Botrytis cinerea* strain (Bo004) was grown on PDA for 10 days at 26±2°C under dark conditions. To recover conidia, Petri dishes were flooded with 20 ml of 0.1% sterile malt extract broth or sterile water containing 0.1% Tween 80 for *in vitro* or *in planta* bioassay, respectively. The suspension was filtered through a sterile mesh and conidial concentration was estimated by using a hemacytometer.

**Extracellular crude fungal extracts**

Cultures were filtered through sterile cloth to remove the mycelium and the filtrate was separated on a Sorvall® Biofuge stratos centrifuge at 10,000 rpm and 8°C for 15 min. The crude fungal extracts were stored at -70°C.

**Antifungal activity in vitro**

*In vitro* *B. cinerea* conidial germination inhibition was tested in 96-well microtiter plates as previously described by Pohanka & collaborators (2004). Briefly, each sample well of a microtiter plate (Corning®, Corning Glass Works) was inoculated with 100 µl of 5×10⁵ conidia/ml and 100 µl of crude fungal extracts. Different controls were considered: wells with 0.1% conidia suspension in sterile malt extract broth and wells containing only the medium or crude fungal extracts. Four replicates were used for treatment. After inoculation, optical density values (λ=405nm) of the individual wells were recorded using a Asys Expert® microplate reader. The conidial germination (expressed in percentage) and fungal growth were monitored after incubation on a rotatory shaker at 100 rpm and 26±1°C for 16 h under dark conditions, this parameter was evaluated by direct observation in an optical microscope (40×) of each well stained with 10 µL of lactophenol blue (to stop growth and to enhance contrast). The experiments were conducted in five blocks on time.

The percentage of germination inhibition was determined with the following equation:

\[
\% \text{Inhibition (I)} = (100 - \%G_{\text{extract}}) - (100 - \%G_{\text{Bo}}),
\]

where: \(%G_{\text{extract}} = \text{percent germination of } B. \text{ cinerea in presence of crude fungal extract and } \%G_{\text{Bo}} = \text{percent germination of } B. \text{ cinerea in absence of crude fungal extract.}

**Detached leaflets bioassay**

Detached leaflets of 6-7 week old of tomato (*Lycopersicon esculentum* c.v. Rocio) with similar sizes from plants grown in a polyethylene greenhouse with no application of fungicides were used according to previous protocol (Meyer et al., 2001). Boxes with 16 leaflets placed with the surface up in a plastic box (24×24×10 cm) with a moistened towel paper in the bottom as experimental unit were incubated at 19 ±/− 1°C for 19 days. The crude fungal extracts selected for its high germination inhibition level (Statgraphics 4.2) were sprayed on leaflets homogeneously (1 ml) by using a manual PVC spray and subsequently were inoculated on leaflet apex with 25 µl of *B. cinerea* suspension (1×10⁵ conidia/ml). The three control treatments comprised leaflets treated only with the pathogen, leaflets with the crude extract and an untreated control.
Statistical analysis
Clustering method of single linkage (nearest neighbor, Statgraphics 4.2) was used to analyze the germination inhibition (I) obtained in the presence of the crude fungal extracts tested. Previously, three clusters were established to compare treatments: low inhibition (1), average inhibition (2) and high inhibition (3). A calibration model (Statgraphics 4.2®) for describing the relationship between percent of conidial germination and fungal growth (absorbance $A_{405}$) was used. Percentage of incidence, severity and efficacy was calculated at 10 and 15 days after inoculation and treatments were compared using Fisher least significance difference technique (LSD) ($\alpha=0.95$; Statgraphics 4.2®).

Results and discussion

Screening of crude fungal extracts in vitro
Fifteen extracts produced on YMG and seven extracts produced on Czapeck-Dox medium, inhibited germination of $B.\ cinerea$ conidia more than 60% as compared with the untreated pathogen control and were clustered on medium and high germination inhibition groups (Figs 1A and 1B). From these, four crude fungal extracts were selected for further testing in planta, since they inhibited $B.\ cinerea$ germination between 85 and 90% after 16h of incubation time. These extracts were obtained from *Penicillium* sp. isolate 44 and *Beauveria* sp. isolate 223 produced on YMG and from *Aspergillus* sp. isolate 110 and *Phialophora* sp. isolate 150 produced on Czapeck-Dox. The characteristics for these extracts are presented in Table 1.

Table 1. Main characteristics of selected fungal extracts for $B.\ cinerea$ control

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Isolate</th>
<th>EFE</th>
<th>Production media (initial pH = 7.0)</th>
<th>Germination inhibition (%), t = 16h</th>
<th>Final pH</th>
<th>Initial color</th>
<th>Final color</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penicillium</em> sp.</td>
<td>44</td>
<td>49</td>
<td>YMG</td>
<td>86.1</td>
<td>4.5</td>
<td>Yellow</td>
<td>Brown</td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td>110</td>
<td>66</td>
<td>CD</td>
<td>90.4</td>
<td>4.5</td>
<td>Colorless</td>
<td>Colorless</td>
</tr>
<tr>
<td><em>Phialophora</em> sp.</td>
<td>150</td>
<td>158</td>
<td>CD</td>
<td>85.3</td>
<td>8.0</td>
<td>Colorless</td>
<td>Black</td>
</tr>
<tr>
<td><em>Beauveria</em> sp.</td>
<td>223</td>
<td>56</td>
<td>YMG</td>
<td>85.6</td>
<td>4.5</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

Different colors and pH characteristics were obtained in extracellular fungal extracts but no relationship was established with $B.\ cinerea$ germination inhibition. When fungal growth was assessed by turbidimetric technique ($A_{405}$), calibration model explaining 64% of the conidial germination of $B.\ cinerea$ obtained in the experimental treatments (data not shown).
Figure 1. Cluster scatterplot of percent of conidial germination inhibition obtained with crude fungal extracts produced on Czapek-Dox (A) and YMG (B) medium. Clusters of inhibition: 1(o) low; 2( ) medium; 3(x) high (+) centroids (Stagraphics 4.2).

**Antifungal activity of the selected crude extracts**

The four previously selected fungal extracts (44, 66, 158 and 56) were used on leaflets bioassay for control of *B. cinerea*. No protection effect was observed with extract 158 while a significant reduction of lesion necrosis caused by *B. cinerea* (74% of reduction) was observed with extract 56 obtained from *Beauveria* sp.

![Figure 2. Progress of severity for B. cinerea in tomato leaflets](image)

In the absence of fungal extracts *B. cinerea* caused necrotic lesions from the apex to all leaflets surfaces. The extract 158 obtained from *Phialophora* sp. negatively affected pathogen germination *in vitro* (85.3%), but not disease development on tomato leaflets (Fig. 2), whereas the extract 56 obtained from *Beauveria* sp. significantly reduced *B. cinerea* germination in 85.6% and disease severity in 74% as compared with the other treatments. More detailed research is needed in this area. Greenhouse assays will revealed whether or not this fungal extracts can be included in integrated control programs for grey mould.
References


Selection of isolates of *Trichoderma* spp. with biocontrol activity over *Rhizoctonia solani* in potato

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**Abstract:** Ten native isolates of *Trichoderma* spp. (Th002, Th003, Th007, Th008, Th015, Th034, Th035, Th172, Th181, Th196) were assessed for their ability to degrade *Rhizoctonia solani* sclerotia. Five *Trichoderma* isolates (Th002, Th003, Th007, Th034, Th181) were selected due to their sclerotia parasitism activity after they colonized more than 50% the *R. solani* sclerotia. When the biological control activity of these isolates was evaluated under greenhouse conditions, *T. koningii* Th003 and *Trichoderma* sp. Th034 reduced damping-off in potato plants by 45.5 and 18.7%, respectively, and significantly increased plant growth.

**Keywords**: biological control, micoparasitic, sclerotia

**Introduction**

The phytopathogenic fungus *Rhizoctonia solani* kühn affects roots, stems and potato tubers, reducing crop yields up to 50% (Diaz, 2002). Traditionally *R. solani* is controlled by using fungicides which is the only means of control currently available in Colombia for this disease. Promising results have been obtained by using species of *Trichoderma* to control this pathogen in potato crops (Tronsmo & Gordon, 1997; Lewis & Lumsden, 2001; Tsror et al., 2001). This study aimed at the selection of native *Trichoderma* spp. isolates with potential biocontrol of *R. solani* in potato.

**Materials and methods**

**Fungal isolates**

The isolate Rh005 of *R. solani* used in this screening was obtained from potato tubers harvested at the CORPOICA’S Research Center located in Cundinamarca (Colombia), previously disinfested and placed in humidity chambers for 7 days at 23°C. The obtained mycelium was inoculated on PDA and incubated for 8 days at 20°C. The inoculum to conduct the biocontrol assays was produced in the culture medium reported by Tsror et al. (2001), and incubated at 24°C for 10 days. Ten isolates of *Trichoderma* spp. (Th002, Th003, Th007, Th008, Th015, Th034, Th035, Th172, Th181, Th196) from the CORPOICA collection were grown on PDA for 7 days at 24°C. A suspension was adjusted to 10⁷ conidia mL⁻¹.

**Sclerotia degradation assays**

Ten sclerotia of *R. solani* Rh005 placed in netting bags (4×5 cm) were buried in plastic pots containing 460 g of 50:50 v/v turf/sand mix (sterile and non sterile mix) at approximately 3 cm below the surface. Biological control agents were then added as a spore suspension (10⁷ conidia g⁻¹). Pots were incubated at 21°C for 15 days, and subsequently sclerotia were
retrieved and assessed for degradation. Then, sclerotia were sterilized in the surface, assessed for viability or for parasitism by squashing on water agar pH 4.5, and incubated for 20 days at 20°C. Twenty replicates of sclerotia test were set up for each isolate of *Trichoderma*. Control treatments consisted on sclerotia where only water was added. The assays were carried out in a completely randomized design. Statistical analyses were performed using ANOVA and Tukey’s test for comparisons of means (P≤0.05).

**Potato seedlings bioassay**

Plastic bags containing soil were inoculated with *R. solani* Rh005 at 2% w/w mycelia/soil. Five days later, the *Trichoderma* spp. isolates (Th002, Th003, Th007, Th034, Th181) selected in the previous experiment were inoculated to obtain a concentration of 10⁷ conidia per g of soil. Potato seedlings cv. “Parda Pastusa” produced *in vitro* were used throughout the experiments. Roots of potato seedlings were immersed in suspensions of *Trichoderma* spp. for 20 minutes (10⁷ conidia. mL⁻¹) before planting. Additionally, suspensions of the *Trichoderma* isolates at the above mentioned concentration were added to soil after 10, 20 and 30 days after planting. The experiment was conducted in a greenhouse using a randomized block design. Statistical analyses were performed using ANOVA and an orthogonal contrast method for comparisons of means (P≤0.05). The experimental unit consisted on 30 plants per treatment with three replicates. A pathogen inoculated control treatment, an un-inoculated controls and a chemical treatment (carbendazim applied at recommended commercial dose) were considered. Potato plants were assessed for symptoms every 10 days up to 40 days.

**Results and discussion**

When sclerotia were buried in the untreated turf/sand mix (sterile or not) 100% were recovered, while when the mix was treated with *Trichoderma* spp., sclerotia germination ranged from 5 to 35%. *Trichoderma* Th007 and Th034 in the sterilized mix significantly parasitized 35% of sclerotia. In non sterile turf/sand mix, no significant differences were observed among treatments. In sterile mix treated with Th181, Th003, Th002, Th007 and Th034 significant differences compared with the untreated control ranging from 60-75% were observed while in non sterile mix only isolates Th034 and Th003 degraded 80 and 85% of the sclerotia, respectively (Table 1). Due to their degradation and germination, isolates with inhibition ability Th034, Th003, Th181, Th002 and Th007 were selected for further bioassays with potato seedlings. In accordance with Dubey & Patel (2001), *R. solani* sclerotia are parasitized and degraded by *Trichoderma*, due to the liberation of enzymatic and antibiotic substances.

In the potato seedlings bioassay four *Trichoderma* isolates isolates; Th003, Th002, Th181 and Th034, significantly reduced damping-off and root rot by 45.5, 36.6, 36.6 and 18.7%, respectively, similar to the chemical control (carbendazim) (Fig. 1). Only Th003 and Th034 increased plant growth expressed as dry weight by 73 and 66%, respectively compared with the untreated control (Fig. 2). These results are in accordance with those established by different authors who also found a relationship between plant growth promotion and the control of pathogens by *Trichoderma* (Windham et al., 1986; Yedidia et al., 1999; Harman et al., 2004), being the growth promotion explained by the improvement of nutrients assimilation by the plant, the production of hormones, and the control of minor pathogens as *R. solani* (Windham et al., 1986).

Results from the greenhouse trial on potato seedlings show that the efficacy of some *Trichoderma* isolates (Th002, Th003, Th007, Th034 and Th181) is equivalent to that achieved with the commercial fungicide based on carbendazim. It can be concluded that it is possible to find biocontrol agents out of a collection of isolates that belong to the genus
Trichoderma. Despite variability among isolates of this collection was observed, this work demonstrated the potential use of T. koningii Th003 and Trichoderma sp. Th034 due to their biocontrol activity and their ability to induce plant growth.

Table 1. Effect of Trichoderma spp. on sclerotia of Rhizoctonia solani (Rh005)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Germinated sclerotia (%)</th>
<th>Sclerotia parasitized by Trichoderma spp. (%)</th>
<th>Sclerotia degraded by Trichoderma spp. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>NS</td>
<td>S</td>
</tr>
<tr>
<td>Th002</td>
<td>5 a</td>
<td>25 a</td>
<td>5 ab</td>
</tr>
<tr>
<td>Th003</td>
<td>5 a</td>
<td>15 a</td>
<td>5 ab</td>
</tr>
<tr>
<td>Th007</td>
<td>30 a</td>
<td>30 ab</td>
<td>35 b</td>
</tr>
<tr>
<td>Th008</td>
<td>10 a</td>
<td>5 a</td>
<td>10 ab</td>
</tr>
<tr>
<td>Th015</td>
<td>15 a</td>
<td>15 a</td>
<td>15 ab</td>
</tr>
<tr>
<td>Th034</td>
<td>35 a</td>
<td>5 a</td>
<td>35 b</td>
</tr>
<tr>
<td>Th035</td>
<td>0 a</td>
<td>20 a</td>
<td>0 a</td>
</tr>
<tr>
<td>Th172</td>
<td>0 a</td>
<td>15 a</td>
<td>5 ab</td>
</tr>
<tr>
<td>Th181</td>
<td>5 a</td>
<td>15 a</td>
<td>5 ab</td>
</tr>
<tr>
<td>Th196</td>
<td>10 a</td>
<td>10 a</td>
<td>10 ab</td>
</tr>
<tr>
<td>Control</td>
<td>85 b</td>
<td>90 b</td>
<td>0 a</td>
</tr>
</tbody>
</table>

S: Sterile turf/sand mix; NS: Non sterile turf/sand mix; Th: Trichoderma. Different letters within columns indicate significant differences between treatments. Tukey test (P≤0.05).

Figure 1. Effect of Trichoderma spp. on control of Rhizoctonia solani (Rh005) expressed as protection of disease (%) in potato seedlings bioassay. Evaluation was done forty days after planting. Orthogonal contrast (P≤0.05).

Figure 2. Effect of Trichoderma spp. on plant growth promotion in the presence of Rhizoctonia solani (Rh005) expressed as dried biomass (g) of potato seedlings. Evaluation was done forty days after planting. Orthogonal contrast (P≤0.05).
Acknowledgement

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References


Biological control of foliar diseases in tomato greenhouse crop in Colombia: selection of antagonists and efficacy tests

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Abstract: The potential biocontrol of isolates *Trichoderma virens* Gl004 and Gl006, *T. koningii* Th003, *Trichoderma* spp. Th034 and Th035, *Clonostachys rosea* Cc001 and the commercial product (TRICHODEX®) based on *T. harzianum* T39 evaluated against *Botrytis cinerea* in tomato stem pieces reduced disease incidence by 50-97% as compared with the untreated control. Isolates Th003 and Cc001 and the product TRICHODEX significantly reduced powdery mildew disease incidence and severity as compared with the untreated control when evaluated against naturally occurring foliar diseases in a commercial unheated greenhouse tomato crop. TRICHODEX inhibited disease incidence in the leaves of tomato plants in 51% whereas *T. koningii* Th003 and *C. rosea* Cc001 reduced disease incidence by 49 and 45% respectively. Both TRICHODEX and *T. koningii* Th003 reduced disease severity by 92% whereas *C. rosea* Cc001 presented 76% powdery mildew severity reduction.

Keywords: biological control agents, gray mold, powdery mildew

Introduction

*Botrytis cinerea* and *Oidium lycopersicum* are important pathogens in tomato greenhouse crops in Colombia (Lee et al., 2001) and the control of these fungi relies entirely on fungicide application. The use of microorganisms for biological control is of great interest due to the undesirable side effects of pesticides. The purpose of our study was to select potential antagonists fungi able to reduce the incidence of *B. cinerea in planta* bioassay, and to evaluate the biocontrol activity of these selected isolates on foliar diseases in tomato greenhouse crop.

Materials and methods

Selection of antagonist fungus in planta bioassay

Antagonists selection bioassay on *B. cinerea* strain Bo004 isolated from naturally infected tomato fruit using stem pieces was conducted according to O’Neill et al. (1996) and Dik et al. (1999) protocols with modifications. Tomato (*Lycopersicon esculentum* cv. Rocio) plants grown in soil in a polyethylene greenhouse were used without application of fungicides. Side shoots of 50 cm in length, from 2 months old plants were collected for this experiment. Ten stem pieces each 4 cm in length as experimental unit, were vertically inserted with the youngest end uppermost into a Styrofoam base, placed in a plastic box (24×24×10 cm) with a moistened towel paper in the bottom and incubated at 22°C.

Potential native biological control agents (BCA) *Trichoderma koningii* Oudemans (Th003), *Trichoderma* spp. (Th034 and Th035), *T. virens* (Gl004 and Gl006), *Clonostachys*...
rosea (Cc001) were selected in previous studies due to their high biocontrol activity on several soil-borne phytopathogens in different plants (Cotes, 2001; Cotes et al., 2001). These native agents together with C. rosea (CcStt) donated by Dr. John Sutton (Guelph University, Canada) and the commercial preparation TRICHODEX 25 WP® Makhteshim Chemical Works Ltd. Israel, based on T. harzianum (T39) were evaluated. The fungi were grown on potato sucrose agar (PSA) and the suspensions of conidia of B. cinerea (1×10^7/mL) and biocontrol agents (1×10^6/mL) were prepared in sterileTween 80 (0.01% v/v) solution from 14 day cultures, whereas TRICHODEX was prepared as a suspension in water (0.1 g/100 mL), equivalent to 1×10^6 CFU/mL. Wounds on stem pieces in the plastic box were inoculated with 2.5 mL of conidial suspension of BCAs by a manual PVC spray (30 cm^3) 8 h before B. cinerea inoculation. Stem pieces were assessed every 2 days starting when the infection symptoms appeared and the extent of tissue rotting and the degree of sporulation of B. cinerea with a scale (0-4) were determined. The infection and sporulation severity indices were calculated for all stem pieces as described by Dik et al. (1999). The proportion of infected stem pieces was also determined and the control efficacy was calculated. The experiment was arranged in a completely randomized design with four replicates per treatment. Data were compared statistically using Fisher’s protected LSD Test at \( P \leq 0.05 \).

**In vivo efficacy test**

Tomato seedlings cv. Rocío were transplanted into soil beds (1.2×20 m) with two rows per bed spaced 0.5 m apart and 0.4 m between plants, under a polyethylene greenhouse conditions. The plots size consisted of 1.2×3 m (7 plants/plot). There were four replications in a split plot with a basic randomized complete block design. The plants were irrigated and fertilized with solid fertilizer 13-26-6% N-P-K applied at the moment of transplanting and one month later. No chemical pesticides were applied. The biocontrol activity of the T. koningii (Th003), C. rosea (Cc001) and the TRICHODEX was measured against foliar tomato diseases caused by fungi. Suspensions of the BCAs (1×10^6 conidia/mL) were prepared and were sprayed weekly when the foliar diseases appeared in the crop. An untreated control was included as well as buffer tomato untreated beds to avoid contamination between treatments. Natural epidemics of O. lycopersicum occurred, with initial symptoms of powdery mildew appearing 99 days after transplant. The disease incidence was determined by counting the infected leaves on the plant and the disease severity was evaluated according to the coverage percentage of the leaves by the mycelium in four different canopy levels at the end of the experiment. Differences in biocontrol activity were determined by using ANOVA for the area under the incidence progress curve (AUDPC) and disease severity. The treatments were compared statistically using Fisher’s protected LSD Test at \( P \leq 0.05 \). Statistical analyses of the data in both experiments were performed using GLM procedure (SAS Institute Inc.).

**Results and discussion**

**Effect of biocontrol agents on botrytis stem rot**

Stem rot caused by B. cinerea was present at the wound in the uppermost part and developed towards other areas. Seven days after inoculation stem pieces inoculated with B. cinerea only and those treated with T. virens isolates and TRICHODEX, developed early Botrytis stem rot with 20, 2.5 and 5% respectively as compared with the other treatments. In stem pieces treated with Trichoderma spp. Th035 and Th003, the infection was delayed eleven days after inoculation, whereas C. rosea Cc001 delayed six days later. Twenty days after inoculation, the incidence of infected stem pieces was significantly reduced by all the tested isolates (\( P \leq 0.05 \)) from 50-97%, obtaining the highest biocontrol efficacy among native isolates with Th034 and Th003 with 97 and 92% respectively and C. rosea CcStt completely inhibited
Botrytis infection (Table 1). The severity of stem rot was significantly reduced only by Trichoderma sp. Th034, whereas the other isolates were not significantly different as compared with the control (Table 1). Botrytis sporulation occurred at the same time than lesions appeared in the majority of the infected stem pieces, but T. virens (Gl004, Gl006) and T. koningii Th003 reduced the sporulation incidence twenty days after inoculation in 25, 16 and 66% respectively. According to these observations the preventive application of all antagonists tested would control the stem rot infection caused by B. cinerea. Since B. cinerea inoculum density in greenhouse environment (Jarvis, 1980) is normally inferior to that utilized in this bioassay, the antagonists evaluated would achieve an effective diseases control when are applied as a preventive treatment.

Table 1. Effect of biocontrol agents on development of stem rot caused by B. cinerea (Bo004)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Gl004</th>
<th>Gl006</th>
<th>Cc001</th>
<th>CcStt</th>
<th>Untreated control</th>
<th>Th003</th>
<th>Th034</th>
<th>Th035</th>
<th>TRICHODEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time&lt;sup&gt;w&lt;/sup&gt; (days after inoculation)</td>
<td>7</td>
<td>7</td>
<td>13</td>
<td>20</td>
<td>7</td>
<td>11</td>
<td>20</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Incidence (%)&lt;sup&gt;x&lt;/sup&gt;</td>
<td>2,5 b</td>
<td>2,5 b</td>
<td>2,5 b</td>
<td>0,0 b</td>
<td>20,0 a</td>
<td>2,5 b</td>
<td>2,5 b</td>
<td>2,5 b</td>
<td>5,0 b</td>
</tr>
<tr>
<td>Incidence (%)&lt;sup&gt;y&lt;/sup&gt;</td>
<td>10,0 c</td>
<td>15,0 c</td>
<td>10,0 c</td>
<td>0,0 c</td>
<td>90,0 a</td>
<td>7,5 c</td>
<td>2,5 c</td>
<td>10,0 c</td>
<td>45,0 b</td>
</tr>
<tr>
<td>Infection severity&lt;sup&gt;z&lt;/sup&gt;</td>
<td>3,7 a</td>
<td>3,2 a</td>
<td>3,5 a</td>
<td>0,0 b</td>
<td>3,5 a</td>
<td>4,0 a</td>
<td>1,0 b</td>
<td>3,5 a</td>
<td>3,6 a</td>
</tr>
<tr>
<td>Sporulation severity&lt;sup&gt;z&lt;/sup&gt;</td>
<td>3,0 a</td>
<td>2,8 a</td>
<td>3,3 a</td>
<td>0,0 b</td>
<td>3,3 a</td>
<td>4,0 a</td>
<td>1,0 b</td>
<td>3,3 a</td>
<td>4,0 a</td>
</tr>
</tbody>
</table>

<sup>x</sup> Values of incidence observed at incubation time
<sup>y</sup> Values of incidence observed at the end of the experiment (20 days after BCA application)
<sup>z</sup> Index of severity calculated at day 20 after BCA application

<sup>w</sup> Period in which the stem rot symptoms appeared

<sup>Period in which the stem rot symptoms appeared</sup>
<sup>x</sup> Values of incidence observed at incubation time
<sup>y</sup> Values of incidence observed at the end of the experiment (20 days after BCA application)
<sup>z</sup> Index of severity calculated at day 20 after BCA application

Values within the same row followed by different letters are significantly different ($P \leq 0.05$) as determined by means of Fisher’s protected least significant difference test (LSD).

Greenhouse experiment
On the basis of the obtained results in planta bioassay, the native isolates T. koningii Th003 and C. rosea Cc001 were evaluated against foliar pathogens in a tomato greenhouse crop with TRICHODEX included as positive control. Since gray mold was observed 99 days after transplanting, the biocontrol treatments were started. However the prevalent environmental conditions during the experiment favored the powdery mildew disease which was more intensive in untreated plots and guard rows than in treated plots. The pathogen infected early old leaves and 27 days after the first application of BCA a significant reduction of powdery mildew incidence was observed as compared with untreated control (Fig. 1A); however, dissemination of the increased disease was observed. When the AUDPC was analyzed, the biocontrol treatments were significantly different as compared with the untreated control, thus TRICHODEX treatment presented the major disease reduction by 51%, Th003 with 50% and Cc001 with 45%. When disease severity was calculated for the whole plant significant differences were observed among treatments and it was reduced by BCAs by 92, 92 and 76%, respectively. When disease severity was evaluated on four canopy levels on the plant, it was observed that at a height of 1.6 m above ground was not significantly different than untreated control, whereas in the other heights evaluated the differences were significant (Fig. 1B).
Similar results were obtained in cucumber in the *Sphaerotheca fusca* - *T. harzianum* T39 system (Elad et al., 1998), in which declination of the control efficacy of the biocontrol agent was related to the age increase of the leaves on the plant.

Figure 1. Effect of biological control agents on powdery mildew disease under greenhouse conditions. A. Incidence progress (infected leaves per plant). B. Leaf area with powdery mildew colonies at 45 days after the first application of treatments. * = Significantly different from control at \( P \leq 0.05 \) as determined by means of Fisher’s protected least significant difference test. The treatments were applied seven times.

**Acknowledgements**

This study was supported by the Colombian Agricultural Research Corporation CORPOICA and the National University of Colombia.

**References**


Ability of the antagonistic bacteria *Bacillus subtilis* and *B. licheniformis* to control *Botrytis cinerea* on fresh-market tomatoes

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Abstract. Grey mould caused by *Botrytis cinerea* is the most important pre-harvest and post-harvest disease on fresh-market tomatoes in Tunisia. Chemical control against grey mould of tomatoes is not effective due to the development of resistant strains as well as the increased concern of consumers towards pesticide use. No fungicides have yet been registered for post-harvest treatment of *B. cinerea* in tomatoes. Biological control based on the use of microorganisms to suppress post-harvest diseases of fruits and vegetables offers an attractive alternative that has gained attention and has shown significant potential (Wisniewski and Wilson, 1992). *Bacillus* spp. offer a great advantage of producing endospores, which are particularly amenable to formulation and long-term storage, and allow these bacterial antagonists to withstand harsh environmental conditions (Fiddman & Rossall, 1995; Powell et al., 1990). In this study, we describe the ability of bacteria of the genus *Bacillus* isolated from different Tunisian soils to protect fresh-market tomato fruits from *B. cinerea*. The tomatoes tested were at two different stages of ripening, (i) mature green and (ii) red. Among 148 bacterial isolates tested on red tomatoes, 6 (4%) significantly reduced growth of the pathogens from 67 to 87%. The effectiveness of these antagonists was also confirmed on green tomatoes; where the fruit rot protection rate ranged from 74 to 100%. The characterization of antagonists was performed by morphological, biochemical and physiological tests as well as 16S rDNA sequencing. The effective isolates were identified as belonging to the species *B. subtilis* and *B. licheniformis*. The biological control potential of these selected bacteria may be correlated significantly with their ability to produce antibiotics and a variety of extracellular hydrolytic enzymes such as chitinases, glucanases and proteases. Our results indicate that the use of bacterial antagonists should be helpful in reducing grey mould disease of tomatoes under storage. A greenhouse assay was also conducted to evaluate the effectiveness of *Bacillus* isolates for controlling grey mould disease in *Planta.*
Suppressive of wheat seedling disease caused by *Fusarium culmorum* using bacterial seed treatment

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**Abstract:** *Fusarium culmorum* is a predominant agent of *Fusarium* head blight (FHB) in cereal crops causing extensive yield and quality losses to wheat in Tunisia. Efforts to manage the disease through the development of resistant cultivars have met with limited success. The agricultural sector that does not use pesticides for control of plant diseases is increasing and there is an urgent need for alternative methods like the use of preparations of microorganisms as biological means of controlling fungal diseases. Selected bacterial isolates of the genus *Bacillus* and *Pseudomonas*, showing antagonism *in vitro* against *F. culmorum*, were evaluated for their ability to suppress Fusarium foot rot under greenhouse conditions. After being treated with a bacterial suspension adjusted at 10⁸–10⁹ CFU/ml, wheat disinfected seeds were sown in soil pots artificially infested with *F. culmorum*. In comparative assays, wheat seeds immersed in a conidial suspension at 10⁵ conidia/ml, were treated with antagonistic bacteria then sown in sterilized soil pots. The reduction of the disease incidence by bacterial antagonists ranged from 79 to 100%; the emergence of seeds was significantly higher in treated pots than in the control pots. Symptoms of the disease were absent in pots treated with *Bacillus subtilis* SR146 or *Pseudomonas aeruginosa*, as compared to the untreated control pots, which showed advanced symptoms of seedling blight. Bacterial antagonists might be good candidates to control the disease under greenhouse conditions. Further evaluation of these antagonists to suppress FHB in the field is recommended.

**Keywords:** biological control, *Bacillus subtilis*, *Pseudomonas aeruginosa*

**Introduction**

*Fusarium* fungi, including *F. culmorum*, *F. graminearum*, *F. poae* and *F. avenaceum*, cause seedling blight, foot rot, and head blight diseases of cereals, resulting in yield loss (Parry et al., 1995). *Fusarium* head blight (FHB) is a major cereal disease worldwide which can result in mycotoxin contamination of grains (Pieterse et al., 2001). Seedling blight and foot rot cause extensive damage to growing seedlings (Wiese, 1977) and lead to a reduction in plant establishment, number of heads per square meter, and grain yield (Humphreys et al., 1998; Wong et al., 1992).

The agricultural sector that does not use pesticides for control of plant diseases is increasing and there is an urgent need for alternative methods. One of these methods is the use of preparations of microorganisms as biological means of controlling fungal diseases (e.g. Weller, 1988; Cook, 1993; Thomashow & Weller, 1996). Many soil and rhizosphere bacterial isolates including fluorescent Pseudomonads, *Pantoea* sp., *Bacillus* sp. and the fungus *Trichoderma harzianum*, have shown promising result in the control of *Fusarium* seedling rot caused by *F. graminearum*, *F. culmorum* blight wheat disease in glasshouse and field studies.
(Bello et al., 2002; Johansson et al., 2003). Many studies have shown that fluorescent
*Pseudomonas, Pantoea* sp., *Bacillus* spp., and *Trichoderma harzianum* are among the most
effective microbes in controlling a range of soilborne diseases (Bernard et al., 1995; Defago
& Haas, 1990; Johansson et al., 2003; Raaijmakers et al., 1998). These and similar results
suggest the possibility of finding new specific bacterial isolates able to suppress disease
development under practical cropping conditions. The objective of this study was to identify
and assess different soil bacteria able to suppress *Fusarium culmorum* responsible for seeding
blight and foot rot of wheat. Isolates were used in treatment of wheat seeds artificially
infected with *F. culmorum*.

**Materials and methods**

**Isolation of antagonistic bacteria and identification**

Bacteria strains used in this paper were isolated from forest soil, agricultural soil and natural
salty soil. To isolate and enumerate soil bacteria, we used three 10 g soil subsamples. A
decimal dilution series was prepared in sterile distilled water up to 10⁻⁵. From each dilution,
0.1 ml aliquots were spread-plated onto TSA (Tryptic Soy Agar, Difco). The plates were then
incubated at 30°C for 72 h. Distinct single colonies were picked and streaked several times to
obtain pure cultures. Strains were then maintained on TSA slants at 4°C and subcultured at
two-month intervals. For long-term storage, strains were conserved in 75% glycerol at 80°C.

The most effective strain was identified using biochemical, physiological and 16S
rDNA sequence analysis method. The 16S rDNA was amplified by PCR using two universal
program was 95°C for 4 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for
1 min, and a final 7 min extension at 72°C. The purified 16S rDNA was sequenced directly by
the dideoxynucleotide chain-termination method (Sanger et al., 1977) using the ABI PRISM
Big Dye Terminator Cycle sequencing.

The inhibitory bacteria were identified using biochemical, physiological and 16S rDNA
sequence analysis method. The 16S rDNA was amplified by PCR using two universal primers
S-D-Bact-0008-a-S-20 and S-D-Bact-1495-a-A-20. The sequences were compared with those
accessioned in GenBank by using BLAST search.

**Fungal isolates and culture conditions**

*Fusarium culmorum* was obtained from the collection of the Laboratory of Mycology of the
Institut National de la Recherche Agronomique de Tunisie (INRAT). This fungus was
routinely grown on PDA medium at 25°C and subcultured onto fresh PDA plates within a
period of 2 months.

**In vitro screening of isolates for antagonism**

Bacterial isolates were screened in vitro against *F. culmorum* by applying a dual culture
technique. *Bacillus* isolates were streaked in a straight line at one side of the Petri dish (3 cm
from the center). Simultaneously, a 5 mm mycelial plug cut from the edge of a 7 day-old
culture of the fungal strain was placed at the center of the plate. After 7 days at 28°C the
inhibitory effect on fungal growth was evaluated. All in vitro antagonism assays were made in
triple.

**Bioassay and testing of bacterial isolates for disease suppressive effects**

To test for potential in vivo bacterial control of foot rot disease in wheat, the effect of six
selected bacteria isolates SR146, OL3, OL11, B3, P'T and PF showing antagonism in vitro on
Fusarium foot rot was assessed using a modified method of Johansson et al., 2003.
Antagonism tests under greenhouse conditions were performed in pots filled with pasteurized
soil, wheat seeds were immersed in conidial *F. culmorum* suspension (1×10⁵ spores/ml, 0.1%
Tween 20), bacterized by bacterial suspension of $10^8$ CFU/ml and then sown in soil pots. *Fusarium* foot rot severity was visually estimated after 16 days.

Table 1. Apparent antagonistic activity of bacterial isolates against *F. culmorum* in vitro

<table>
<thead>
<tr>
<th>Isolate</th>
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<tr>
<td>Forested soil Nahli</td>
<td></td>
<td>Sabkha of Raoued</td>
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<tr>
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<td>0</td>
<td>SR80</td>
<td>0</td>
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</tr>
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<td>P'T</td>
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<tr>
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<td>Rol11</td>
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*Percentage of growth inhibition (IG) based on Whipps’ (1987) formulation. Three categories were represented: 0=no inhibition, 1= 1 à 25%, 2= 26 à 50%, 3= 51 à 75%.

**Results**

*Isolation and screening of soil bacteria*

A total of 69 bacteria were isolated from Tunisian soils. The result of the *in vitro* dual culture screening revealed that 21 of these bacterial isolates reduced the mycelial growth of *Fusarium*
culmorum by forming inhibition zone (Representative results of these bacteria isolates are listed in Table 1). Notably, isolates SR146, SR147, SR3,OL3, P’T, PF and OL11, showed the highest apparent antagonistic activity against *F. culmorum* (Fig. 1). Because of their apparent super antifungal activity, SR146, SR3,OL3, P’T, PF and OL11, were selected for further characterization and investigation.

**Characterization and identification of selected antagonists**

Bacterial identity was determined using morphological and biochemical characteristics (motility, aerobic growth, Gram stain, catalase, oxidase). To confirm the identity of these isolates 16s rDNA gene was sequenced and subjected to BLAST searches. The isolates were identified as belonging to *Bacillus subtilis* (SR146), *Pseudomonas aeruginosa* (OL3), *Pseudomonas* spp. (PF), *Brevibacterium halotolerans* (SR3), *B. cereus* (OL11) and P’T unidentified.

![A](image1.png) ![B](image2.png)

**Antagonism tests under green house conditions**

Based on their performance in the *in vitro* antagonism, six bacterial isolates were included in the inoculation seedling foot rot tests. Control plants treated with bacterial culture exhibited no disease symptoms and appeared healthy, SR146 *Bacillus subtilis*, OL3, *Pseudomonas aeruginosa*; P’T, *Pseudomonas* spp.; SR3, *Brevibacterium halotolerans*; OL11, *Bacillus cereus*; all significantly reduced *Fusarium* seedling foot rot disease symptoms on wheat (Figs 2-3). *Pseudomonas aeruginosa* OL3 was more effective in reducing disease than *Bacillus subtilis* SR146, relative to control plants inoculated with *F. culmorum*. *Pseudomonas* spp. strain PF had no significant effect on the visual symptoms observed on wheat artificially infested with *F. culmorum* (Fig. 2). In pots non infested by the pathogen, seeds treated with bacteria isolates SR146, OL3, B3, OL11, P’T and PF started germination three days before the germination of natural seeds. Plants of wheat treated with bacteria isolates grew more than natural seedlings (Fig. 4).
Discussion

In vitro agar assay as an initial selection method for putative microbial antagonists is a preferred and practical means of screening a large number of isolates for antibiosis. Plant tests, however, remain essential for verifying the effectiveness of potential candidates, as in vitro antibiotic activities often do not correlate with in vivo biocontrol activities (Fravel, 1988). As a model, all bacteria isolates except PF isolate were demonstrated to control in planta the Fusariose disease caused by F. culmorum. Moreover, it was also found in repeated pot experiments to control foot rot in wheat seeds infested with F. culmorum. Such biocontrol with the direct application of live cells to plants presumes their eventual in planta establishment and that the same antibiotics are produced and responsible for disease control as during in vitro bioassays.

Biocontrol organisms including Bacillus subtilis, Pseudomonas aeruginosa, Brevibacterium halotolerans and Bacillus cereus also demonstrated varying degrees of efficacy in reducing Fusarium foot rot of wheat. The most effective of these isolates were B. subtilis SR146 and Pseudomonas aeruginosa OL3. Indeed, of the bacterial biocontrol agents, the genetically diverse bacilli are well-known to possess antagonistic activities against many soilborne fungal and bacterial plant diseases (Handelsman & Stab, 1996). These activities are often purportedly a result of antibiosis elicited by a wide range of antifungal metabolites, including antibiotics, hydrolytic enzymes, and proteases produced in vitro (Katz & Demain, 1977) and in vivo (Fravel, 1988). In fact, purification and characterization of the antifusarial substance(s) produced by B. subtilis SR146 from culture filtrate revealed a peptidic nature of substance(s) responsible for inhibition of this fungus.

A general conclusion from the results obtained is that seed treatment with naturally selected bacterial isolates has the potential to induce significant suppression of wheat seedling disease induced by F. culmorum. Thus, now we have identified several organisms as potential antagonists. We are continuing to work with these strains to determine the specific mechanisms, interactions and to test their behaviour under field conditions.
Figure 3. Disease symptom development on wheat inoculated with *Fusarium culmorum* C27 and either bacteria. Visual symptoms were assessed 16 days after inoculation. Seedling foot rot disease symptoms were scored as lesion color (lesion color scale: 1, no disease; 2, very slight brown necrosis; 3, slight/moderate brown necrosis; 4, extensive brown necrosis and plant faded; 5, extensive black necrosis plant dead. Bacterial codes: SR146, *Bacillus subtilis*; OL3, *Pseudomonas aeruginosa*; P'T, *Pseudomonas* sp; SR3, *Brevibacterium halotolerans*; OL11, *Bacillus cereus*; PF, *Pseudomonas* sp.

Figure 4. (a) Seed treated with bacteria, (b) Seed not treated with bacteria

References


Post-harvest biological control of grey mould rot on strawberry fruits using moderately halophilic bacteria

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Abstract. Strawberry fruit have a very short postharvest life, due to grey mold caused by Botrytis cinerea. Control of B. cinerea is normally carried out by the application of fungicides. However, problems related to the use of fungicides exist. Fungicide resistance has been reported in B. cinerea (Washington et al. 1992) and there is a public concern related to the use of fungicides. These problems support the need for alternative methods, which must be safe and able to partially replace fungicide treatments (Wilson & Wisniewski, 1989). Therefore, the search for biological control agents has been intensified in recent years and several microorganisms with high activity have been identified (Janisiewiez, 1988; Peng & Sutton, 1991). The aim of this study was to investigate the possibility of post-harvest biological control of B. cinerea on strawberry fruits using moderately halophilic bacteria isolated from Tunisian Sebkhas. The strawberry fruits were sprayed with the antagonist cell suspension (10⁸ CFU/ml) and then 1 h later with a conidial suspension (1×10⁴/ml) of B. cinerea. The fruits were examined for incidence of the disease after storage at 15°C, for 3 to 4 days. Among 60 moderately halophilic bacteria tested, 11 (18.3%) inhibited efficiently the development of grey mould on strawberry fruits. The reduction of disease incidence was ranged from 30 to 100% after 3 days of storage. 55% of the effective isolates showed antifungal activity more than 40% after 4 days of storage. The most effective isolates were identified by 16S rRNA gene sequencing with conventional phenotypic tests as Bacillus pumilus, B. subtilis, B. marismortui, Virgibacillus marismortui, B. licheniformis and Halomonas sp. These halotolerant bacteria may be considered as halophilic since they grew in media containing 0.5 to 15% NaCl. These strains are a source of hydrolytic enzymes such as chitinases, proteases, laminarinases, lipases and cellulases. Correlation between the ability to suppress the postharvest disease and to produce extracellular antifungal enzymes, together by moderately halophilic bacterial isolates was clearly established. Further studies are needed to correlate between production of bioactive compounds and the concentration of salt in the medium.
Commercial applications of Shemer for the control of pre- and post-harvest diseases

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Abstract: Shemer is a biofungicide based on the yeast Metschnikowia fructicola. The mode of action of this yeast is believed to be mainly through competition, with no involvement of antibiotics or toxins thus creating a minimal impact on the environment. The commercial product (water dispersible granules) is stable under ambient storage conditions, and can be applied through spray or drench application systems in the field or in packing-houses. Shemer treatments in commercial packing-houses significantly reduced the development of Botrytis cinerea on pepper and tomatoes, and of Rhizopus stolonifer on sweet potatoes and peaches. Application of Shemer in the field proved useful also in post-harvest protection of fruits like raspberry, in which post-harvest treatments are not practiced. These results demonstrate the suitability of Shemer for a wide range of crop-pathogen situations, agricultural practices and climatic conditions.

Introduction

New and tighter regulations of pesticide use, as well as consumer awareness have led to the banning of many pesticides and to the search for safer alternatives. The use of microorganisms is considered as one of the preferred options for rot control and numerous microorganisms were identified as potential biocontrol agents (Drobi et al., 2000).

Shemer is a commercially available biofungicide based on the yeast Metschnikowia fructicola. The mode of action of this yeast is through competition for nutrients and space, with no secretion of antibiotic or toxic substances, thus creating minimal effect on the environment. Shemer (water dispersible granules) is stable under ambient conditions, and can be applied through spray or drench application systems. Thus it can be easily integrated in practical crop protection. The utility of Shemer in the prevention of rots in citrus fruit, grapes and strawberries was described previously (Keren-zur et al., 2002). In this paper we focus on the effectiveness of Shemer under commercial field and packinghouse conditions, against several pathogens of sweet-potatoes, pepper, peaches, tomatoes and raspberries.

Materials and methods

Shemer is produced by submerged fermentation of the yeast M. fructicola, followed by formulation into dry, water dispersible granules. The product is manufactured by Agro-Green, the Biological Div. of Minrav, Ashdod, Israel.
**Trial design**

Pre-harvest application was tested in commercial farms. Each treatment consisted of 4-6 replications, each one of 5-8 m row (tomatoes and raspberries) or 5 trees (peaches). Application was done one day before harvest. 4-6 boxes were harvested from each plot.

Post-harvest treatment was applied during the commercial packing process between the washing and the drying steps. Shemer was applied by conventional spraying equipment. Unless stated otherwise, the dosage was 2 gr/liter. Storage conditions were according to the recommendations for each crop. Cumulative incidence of infected fruits (%) was recorded.

**Statistical analysis**

Differences between treatments were determined using a non-paired \( t \)-test (at \( P<0.05 \)) or ANOVA using the program Jump 5.1. Means were compared using the LSD test.

**Results**

**Post-harvest application**

Efficient control of post-harvest rot can be achieved by packing-house treatment, where application of spray is focused on the fruit and difficulties due to environmental exposure are minimized. Shemer integrates into packing practices, by the inclusion of spraying station in line. Shemer is best applied after a washing step and before waxing and drying. The combination of fruit, pathogen, local practices and equipment availability in the specific packinghouse will determine the treatment protocol.

![Figure 1. Control of Botrytis on Pepper during storage. Pepper was brushed with cold water before Shemer application. Storage was at 7°C, RH 95% (S36-05)](image1)

![Figure 2. Control of Rhizopus rot on Sweet potatoes during storage. Sweet-potatoes were brushed with cold water before Shemer application. Storage was at 11°C, RH 90% (S25-03)](image2)
The efficacy of post-harvest Shemer application in citrus fruit has been described previously (Keren-zur et al., 2002). Harvested sweet potatoes are stored for several months before washing and packing. Shemer treatment in the packing line provided effective control of Rhizopus (Fig. 2). The results in Figs 1 and 2 show the efficacy of Shemer in the control of Botrytis in pepper, and of Rhizopus in sweet-potatoes respectively.

**Pre-harvest application**

Due to its safety, Shemer can be applied one day before harvest. The efficacy of pre-harvest Shemer application in table grapes and on strawberries has been described previously (Keren-Zur et al., 2002).

Control of Rhizopus in peaches was tested in two ways- by pre-harvest field application compared with packing line treatment. As can be seen in Fig. 3, the efficacy of the packing line treatment was higher than that of the field application. It is unclear at this stage whether the lower effect of field application is due to poor coverage of the fruit or due to the deterioration of Shemer caused by exposure to the environment. It can be concluded that whenever practiced, the post-harvest Shemer treatment is preferable.

Tomatoes are infested in the field by Botrytis and develop gray mold during storage on fruit as well as on the sepals. As can be seen in Fig. 4, pre-harvest application of Shemer reduced significantly the post-harvest gray mold on the tomatoe sepals.

Post-harvest treatment might cause damage to sensitive fruit such as raspberries. In this case, pre-harvest field treatment with Shemer reduced the development of post-harvest rot (Fig. 5).

![Figure 3](image1.png) **Figure 3.** Control of Rhizopus rot on Peaches during storage. Peaches were brushed with cold water before Shemer application. 6 days storage at 20°C (25-05)

![Figure 4](image2.png) **Figure 4.** Control of Botrytis on Cherry Tomatoes sepals during storage. Storage at 12°C, RH 90% (S15-03)
Conclusions

Shenmer application may be tailored to fit crop and local practices. It is suitable for pre-harvest (field) or post-harvest (packing-house) application, using conventional equipment and practices. The results presented show that Shenmer is effective against several pathogens including *Botrytis* and *Rhizopus*.

Effective prevention of rots was demonstrated on a variety of crops including cherry tomatoes, peaches, sweet potatoes and pepper. The combination of "SHEMER"'s effectiveness and safety provides a real alternative to currently used chemical fungicides.

References

Selection of *Trichoderma* spp. isolates to control the bean white-mold fungus *Sclerotinia sclerotiorum* in winter crops

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**Abstract:** Bean white mold is a destructive disease on autumn-winter crops in Brazil, when daylight length is short and the temperature vary from 15 to 25°C. Chemical control is expensive and can be poorly efficient when applied alone. Several *Trichoderma* species are natural antagonists to *S. sclerotiorum* sclerotia in soil. However, in general the development of the isolates applied as biocontrol agents in Brazil are favoured by temperatures above 25°C. In this case, the use of these isolates on autumn-winter crops can be not efficient. The objective of this work was to select *Trichoderma* spp. isolates able to parasitize the pathogen sclerotia in lower temperatures. In the pot experiment, twenty isolates of *Trichoderma* spp. and one of *Clonostachys rosea* were evaluated. Sclerotia were buried in soil and the following treatments were applied: check; *Trichoderma* spp. isolates (300 L/ha suspension volume at 10⁷ conidia/mL); and, fungicide (cerconil, recommended dose). After five days at 20±2°C, the sclerotia were removed from soil and transferred to carrot slices over water-agar medium. The number of germinated and parasitized sclerotia was assessed after 10 days. The experiment was conducted twice in a completely randomized design with seven replications. In the micro-plots experiment, two isolates of *Trichoderma* spp. and one of *C. rosea* were evaluated. The treatments (antagonists, fluazinam, and water check) were applied weekly from 20 days after plants emerging until the beginning of pods maturation. The experiment was conducted in a randomized block design with three replications. In the pot experiment, the isolates ALF111 and ALF409 consistently inhibited the germination and parasitized more than 80% of the sclerotia. Besides these, the isolates ALF02, ALF57, ALF66, ALF324 and ALF402 were efficient too. The isolate 172H inhibited significantly germination, but it was not capable to parasitize the sclerotia, which suggests that other biocontrol mechanisms, such as antibiosis, are involved. Although there were no visible symptoms of the white mold disease in the micro-plot experiment, the yield in the treatments with *C. rosea*, ALF66 and *Trichoderma* were superior to the check plots. The selected isolates are potential biocontrol agents against bean white mold and will be tested in field conditions.

**Key words:** biological control, *Phaseolus vulgaris*, micoparasitism, common bean

**Introduction**

White mold, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most destructive diseases of common bean (*Phaseolus vulgaris* L.) on irrigated autumn-winter crops in Brazil, when daylight length is short and the temperatures vary from 15 to 25°C (Paula Jr. & Zambolim, 2006). Fungicide sprays to prevent white mold are the standard practice in Brazil, but it is expensive and, frequently, not economically feasible. Beside that, because of environmental considerations, new disease management strategies are needed. The use of
biological control agents to reduce the inoculum of *S. sclerotinia* in soil is a promising alternative (Gerlagh et al., 1999).

Numerous fungal mycoparasites are reported as biological control agent (BCA) against sclerotia of the pathogen in soil, including several *Trichoderma* species (Whipps & Budge, 1990). In warmer times of the year *Trichoderma* spp. are frequently found associated with sclerotia in soil, especially on no-till crops in Brazil (Arancibia et al., 2001). In general the development of these isolates are favoured by temperatures above 25ºC. In this case, the use of these isolates on autumn-winter crops can be not efficient (Bernardes, 2006). The objective of this work was to select *Trichoderma* spp. isolates adapted to winter conditions in Brazil and able to control the bean white-mold in such crops.

Material and methods

**Pot experiments**

Twenty isolates of *Trichoderma* spp. and one of *Clonostachys rosea* were evaluated. Sclerotia of *S. sclerotiorum* produced in carrots-corn media were buried on soil in 3 l pots and the following treatments were applied: check; *Trichoderma* spp. isolates (300 l ha⁻¹ suspension volume at 10⁷ conidia ml⁻¹); and, fungicide (cerconil, recommended dose). After five days at 20±2ºC, the sclerotia were removed from soil and transferred to carrot slices over water-agar medium. The number of germinated and parasitized sclerotia was accessed after 10 days. The experiment was conducted twice in a completely randomized design with seven replications.

**Micro-plots experiments**

Bean (cv. ‘Talismâ’) was grown in one-square meter micro-plots previously infested with *S. sclerotiorum* sclerotia. The treatments consisted of two isolates of *Trichoderma* spp. (Trichode and ALF66), one isolate of *C. rosea*, fungicide sprays (fluazinan) and a check plot. The treatments were applied weekly from 20 days after plants emerging until the beginning of pods maturation. Disease incidence (percentage of diseased plants) was monitored during the complete crop cycle. The final stand and grain yield were evaluated at harvest time. The experiment was conducted in a randomized block design with three replications.

Results and discussion

Significant differences among the *Trichoderma* isolates were found. The isolates ALF111 and ALF409 consistently inhibited the germination and parasitized more than 80% of the sclerotia in the pot experiment (Figs 1 and 2). Beside these, the isolates ALF02, ALF57, ALF66, ALF324 and ALF402 were efficient too. The isolate 172H inhibited significantly germination, but it was not capable to parasitize the sclerotia, which suggests that other biocontrol mechanisms, such as antibiosis, are involved. Isolates ALF69, ALF70, ALF77, ALF1114 and TCNYG were not only ineffective, but stimulated sclerotia germination. Similar results with a *Trichoderma* spp. isolate were reported previously by Gerlagh et al. (1999).

Although there were no visible symptoms of the white-mold disease in the micro-plot experiment, the yield in the treatments with *C. rosea*, ALF66 and Trichode were superior to the check plots (Table 1). No reason for this effect can be advanced. Additional studies to evaluate this effect are warranted. The selected isolates in these preliminary studies are potential biocontrol agents against the bean white-mold. The isolates will be morphologically, biochemically and molecularly characterized and tested in field conditions.
Figure 1. Germination of *Sclerotinia sclerotiorum* sclerotia. The sclerotia were buried in soil (0.5cm) treated with different isolates of *Trichoderma* spp. or fungicide for five days, transferred to carrot slices over water-agar medium and kept at 20±2°C for 12 days. Bars are means followed by standard error. Bars marked with an (*) are statistically different from check (pLSD; *P*=0.05).

Figure 2. Sclerotia of *Sclerotinia sclerotiorum* parasitized by *Trichoderma* spp. isolates. The sclerotia were buried in soil (0.5 cm) treated with different isolates of *Trichoderma* spp. or fungicide for five days, transferred to carrot slices over water-agar medium and kept at 20±2°C for 12 days. Bars are means followed by standard error. Bars marked with an (*) are statistically different from check (pLSD; *P*=0.05).
Table 1. Bean yield in micro-plot experiment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nº of plants/m²</th>
<th>Yield (kg/ha)</th>
<th>Weight of 100 seeds (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>18.7</td>
<td>2842.92 ab</td>
<td>30.88</td>
</tr>
<tr>
<td>S. sclerotiorum</td>
<td>18.7</td>
<td>2624.22 b</td>
<td>31.60</td>
</tr>
<tr>
<td>Fluazinam</td>
<td>18.0</td>
<td>2653.45 b</td>
<td>29.95</td>
</tr>
<tr>
<td>Trichode</td>
<td>18.7</td>
<td>3175.40 ab</td>
<td>29.32</td>
</tr>
<tr>
<td>C. rosea</td>
<td>18.7</td>
<td>3792.80 a</td>
<td>29.97</td>
</tr>
<tr>
<td>ALF66</td>
<td>18.0</td>
<td>3022.22 ab</td>
<td>31.81</td>
</tr>
</tbody>
</table>

Means followed by the same letters are not significantly different (pLSD; $P=0.05$).

Acknowledgements

FAPESP (‘Fundação de Amparo à Pesquisa do Estado de São Paulo’).

References


In vitro effect of cyanobacteria and algal preparations on 
*Colletotrichum lagenarium* and on the fungal-cucumber 
interaction

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Abstract: The cyanobacteria and algae are potential biological control, and the anthracnose is one of the 
most important diseases on cucumber. The objectives of the work were to study the effects of algal 
preparations on mycelial growth, conidium germination and appressorium formation by *C. lagenarium* 
and to study the effects of preparations in the expression of disease symptoms in cucumber seedlings. 
The treatments were represented by cell suspensions, intra-cellular content and the culture medium 
filtrate from the cyanobacteria *Nostoc* sp. 21 and *Nostoc* sp. 61 as well as from the algal strain 067/02. 
The controls were represented by distilled water, BG11 medium and the resistance inducer acibenzolar-S-methyl. The *in vitro* assays for mycelial growth were carried out on oat-agar medium, 
and the results showed that all the suspensions inhibited growth. For spore germination and 
appressorium formation evaluation, the preparations and *C. lagenarium* spore suspension were added 
inside wells of ELISA plates. After incubation, the results showed that some of the treatments reduced 
spore germination while the intracellular content stimulated it. On the other hand, the intracellular 
content and the cell suspensions stimulated appressorium formation while de culture filtrate reduced it. 
For *in vivo* assays, one cotyledonary leaf from cucumber seedlings (*Cucumis sativus*) was treated with 
one of the preparations. The other cotyledonary leaf was treated with distilled water. After 24 h, a 
spore suspension from *C. lagenarium* was sprayed onto the two pre-treated cotyledonary leaves. It was 
found that the filtrates from the culture medium reduced locally as well as systemically disease 
symptoms on the seedlings. On the other hand, leaves were also treated and 24 h later inoculated with 
*C. lagenarium* spores. Fifteen hours after inoculation, the leaves were harvested, bleached, stained, 
and observed under microscope. The results showed that the culture medium filtrates as well as the 
susensions were able to reduce *in vivo* spore germination while appressorium formation decreased or 
did not differ from the control treatments, depending upon the preparation used. It can be concluded 
that these microbial agents exhibit potential to control *C. lagenarium* and maybe other fungal plant 
pathogens.

Key words: biological control, induced resistance, anthracnose

Introduction

Several researchers are interested in alternatives to chemical control of phytopathogens 
(Hammerschmidt et al., 2001). Biological control and induced resistance are two alternative 
control measures for pathogens. The induced resistance consists in the activation of latent 
defense mechanisms of a host plant, by treating it with inducers, which will lead to increased 
resistance (Hahn, 1996). This kind of technology can be used in a integrated program for 
plant disease control and contributes to the maintenance of plant genotypes with desirable 
agronomic value (Di Piero & Pascholati, 2002). According to Cannel (1993), the 
cyanobacteria and algae comprise the major biomass of the world. Because of their ability to
produce different toxic compounds against bacteria and fungi, and be cultivated in large scale, they are potential biocontrol agents of phytopathogens (Kulik, 1995). In previous studies, it was verified that preparations from *Synechococcus leopoliensis* e *Nostoc* sp. reduced the number of TMV lesions on tobacco plants (Di Piero et al., 2000), and interfered in the interaction *Colletotrichum sublineolum* – sorghum plants (Di Piero & Pascholati, 2002). The preparations from *S. leopoliensis* and *Limonotrix planctonica* were also able to elicit the accumulation of phytoalexins in sorghum tissue (Di Piero & Pascholati, 2002; Pascholati, 1998). The anthracnose, caused by *Colletotrichum lagenarium*, is one of the most important diseases on cucumber (*Cucumis sativus*). Thus, the objectives of the work were to study *in vitro* the effects of the cyanobacteria and algal preparations on *C. lagenarium* and the effects of the preparations in the expression of symptoms caused by the pathogen in cucumber seedlings.

**Materials and methods**

*Algal isolates, growth and preparations*

Isolates *Nostoc* 21 and *Nostoc* 61 were supplied by Dra. Marly F. Fiori (CENA/USP, Piracicaba, SP, Brazil), and the Isolate 067/02 was obtained from soil samples (Itaquieri region, Charqueada, SP, Brazil). The isolates were grown at 28°C under constant light inside 250 ml erlenmeyers with 100 ml BG 11 liquid medium. Transfers to the new medium were carried out every 21 days. The algal preparations were represented by cell suspension, the filtrate from the culture medium and by the intracellular content (obtained by powdering the cells in liquid nitrogen).

*Pathogen isolation and growth*

The fungus *C. lagenarium* was isolated from cucumber leaves and maintained in oat-meal agar medium under UV-light (long wavelength) at 20°C. Conidia suspensions were obtained after 7 days by adding sterile distilled water onto the medium, and the concentration was adjusted to $1 \times 10^5$ conidia/ml.

*In vitro conidium germination, appressorium formation, and mycelial growth*

Elisa plates were used to evaluate conidium germination and appressorium formation, and aliquots of the preparations (40 µl) and from the spore suspension ($10^5$ conidia/ml) (40 µl) were placed together inside each well. The Elisa plates were maintained at 25°C under constant fluorescent light, and after 24 h lactoglicerol (50%) was added and the number of germinated spores and appressorium formed counted. The mycelial growth experiment was carried out by using Petri dishes with oat-agar medium, where 1 ml of each preparation was spread onto the medium and a mycelium disc (0.6 cm diameter) added in the center. The dishes were incubated at 25°C under fluorescent light (12 h photoperiod), and the evaluations carried out every 2 days.

*Seedling production, algal treatments and pathogen inoculation*

Cucumber seedlings (cv. Caipira Verde) were produced under greenhouse conditions inside small pots, and used when the cotyledonary leaves were fully expanded. One leaf of the pair was treated with the algal preparation by using a brush, and each treatment had 5 replicates. Control treatments were represented by BG 11 medium, distilled water, and the resistance inducer acibenzolar-S-methyl (5 g/l). After 24 h, a spore suspension of *C. lagenarium* ($10^5$ conidia/ml) was sprayed onto all the leaves, and the evaluation of lesioned area carried out 7 days after the inoculation.
In vivo conidium germination, and appressorium formation
Cucumber plants were used when at least 3 true leaves were fully expanded. The leaves were treated with the algal preparations by using a brush, and 24 h later inoculated with C. lagenarium (10^5 conidia/ml). The control treatments were represented by the BG 11 medium, and distilled water. Fifteen hours after the inoculation, the leaves were harvested, placed inside of a glass box, and bleached by using chlorine gas (produced by the reaction of sodium hypochlorite and sulfuric acid) for 15 min. Then, the leaves were stained with lactophenol for 30 s, washed in 1% potassium hydroxide, placed in 1% fucsin for 3 min, washed with 1% potassium hydroxide, placed in 1% Congo red for 3 min, and finally washed with 1% potassium hydroxide. The observation was carried out under an optical microscope.

Results and discussion
The results showed that the cell suspensions had an inhibitory effect on in vitro spore germination (mainly the suspension from isolate 067/02) (Fig. 1), and on mycelial growth (data not shown). The filtrate preparations reduced in vitro appressorium formation, but the intracellular content preparations stimulated in vitro conidium germination and appressorium formation (Fig. 1).

Figure 1. In vitro effect of suspension (S), intracellular content (C) and the culture medium filtrate (M) from the cyanobacteria *Nostoc* sp. 21 (N21) and *Nostoc* sp. 61 (N61) and from the algal Isolate 067/02 (067) on *C. gloeosporioides* conidium germination (left) and appressorium formation (right). The controls were distilled water (H2O) and BG11 (liquid medium).

Mean ± SD.

In in vivo assays, the culture medium filtrates from *Nostoc*-21 and *Nostoc*-61 and the cell suspension preparation from 067/02 significantly reduced conidium germination (Fig. 2). Regarding appressorium formation, only the culture medium filtrate from *Nostoc*-21 significantly reduced it. The culture medium filtrates reduced the disease severity in the *C. gloeosporioides*-cucumber interaction (Fig. 3). Thus, these microbial agents exhibit potential to control in vitro and in vivo the anthracnose pathogen.
Figure 2. *In vivo* effect of suspension (S), intracellular content (C) and the culture medium filtrate (M) from the cyanobacteria *Nostoc* sp. 21 (N21) and *Nostoc* sp. 61 (N61) and from the algal Isolate 067/02 (067) on *C. gloeosporioides* conidium germination (left) and appressorium formation (right) *in vivo*. The controls were distilled water (H2O) and BG11 (liquid medium) Mean ± SD.

Figure 3. Local and systemic effect of suspension (S), intracellular content (C) and the culture medium filtrate (M) from the cyanobacteria *Nostoc* sp. 21 (N21) and *Nostoc* sp. 61 (N61) and from the algal Isolate 067/02 (067) on the anthracnose severity on cotiledonic leaves of cucumber. The controls were distilled water (H2O), BG11 (liquid medium) and Acibenzolar-S-Methyl (ASM). Mean ± SD.

**Acknowledgements**

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**References**


**Effect of soil treatments in the development of strategies for the control of kiwifruit wood decay**

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**Abstract:** The effect of different soil treatments, including i) commercial formulations of *Trichoderma harzianum* strain T22 (Rootshield), ii) a mixture of bacteria and fungi (Ekoprop) and iii) silicon, on physiological and morphological parameters of potted kiwifruit plants artificially inoculated with *Fomitiporia mediterranea* (*Fom*) and *Phaeoacremonium aleophilum* (*Pal*), has been investigated in experiments performed in 2005 and 2006. Glucanase and chitinase activity of leaf proteins, shoot dry weight, leaf chlorophyll content and root apparatus development have been detected. Glucanase activity, determined in 2006, was increased by Ekoprop and Rootshield, *Fom* inoculation, Rootshield + *Pal* inoculation in July and by all treatments + *Fom*, except for silicon in April, and reduced by silicon treatment applied singly. Chitinase activity, detected in 2006, was increased by all single treatments and inoculations, by Rootshield + *Pal* in April, by silicon + *Pal* in June and by Rootshield + *Fom* in April. In 2005, all treatments increased shoot dry weight, whereas silicon caused a slightly decrease in 2006. In general, the chlorophyll content was not influenced by biological treatments, while the root apparatus was enhanced by the treatments.

**Key words:** biological treatments, induced resistance, PR proteins, silicic acid, wood disease

**Introduction**

The decay of kiwifruit is a complex wood disease widespread in Italy, caused by several fungi including *Fomitiporia mediterranea* M. Fisch. and *Phaeoacremonium* spp., which are responsible for leaf necrosis, shoot wilt, yield loss and, in some cases, plant death. At present, an effective control strategy is not yet available. As for “esca” of grapevine, the decay of kiwifruit has a chronic development and is characterized by a complex and prolonged relationship with the host-plant, producing different effects on plant physiology (Mugnai et al., 1999). Moreover, ongoing studies show possible correlations between pathogens associated with the disease and microelements, iron in particular (Di Marco et al., 2001). Therefore, correlations between these pathogens and plant nutritional status may be hypothesized. Besides, also biocontrol agents and silicon are able to effectively interfere with plant physiology making plant less sensitive to pathogen attack and abiotic stress (Chérif et al., 1994; Harman et al., 2004). The aim of this research was to test the effect of different soil treatments, including i) commercial formulations of *Trichoderma harzianum* strain T22, ii) a mixture of bacteria and fungi and iii) silicon, on physiological and morphological parameters of potted kiwifruit plants artificially inoculated with wood decay pathogens.
Materials and methods

Micropropagated plants (cv. Hayward) grown in 1 L volume pots for 4-5 months, were successively transplanted in 5 L pots containing calcareous soil collected from a kiwifruit orchard. Plants were submitted to the following soil treatments: *Trichoderma harzianum* strain T22 (Rootshield®, Intrachem, Italy, 2 g/plant), a soil amendment constituted of a mixture of bacteria and fungi (Ekoprop, CCS, Aosta, Italy, 2 g/plant) and silicon as silicic acid (0.35 g Si kg⁻¹, 1 application at transplanting). The two biological treatments, Rootshield and Ekoprop, were applied two times in 2005 (May and July) and two times in 2006 (April and June). Each treatment consisted of 20 plants (replicates) adequately irrigated and fertilized.

Pathogens, *F. mediterranea* (*Fom*) and *P. aleophilum* (*Pal*), were inoculated in 2 year old plants, in February 2006. A mycelial agar plug of each fungus culture was inserted in a hole made in the trunk, whereafter wounds were covered with a lubricant and wrapped with Parafilm to prevent drying.

Partially expanded apical leaves were excised and rapidly frozen in January (before pathogen inoculation), April, June and July 2006. Leaf proteins were extracted, quantified and tested for their glucanase and chitinase activities by using the gel diffusion assay (Barbagus et al., 2004). Gels were amended with laminarin and glycolchitin for glucanase and chitinase assays, respectively. After the assays, gels were photographed under visible or UV light for glucanase and chitinase determinations respectively, the images were loaded into the image analysis software (Quantity One, BioRad) and the intensity of the activity areas was determined.

The shoot dry weight as well as the content of leaf chlorophyll were periodically assessed, the last one by means of the portable instrument SPAD 502 (Minolta). The development of plant roots was evaluated by video image analysis, before pathogen inoculations, in January.

Results and discussion

The glucanase activity of plants treated with Ekoprop increased, whereas it was progressively decreased by Rootshield application, even though values remained higher than those of the untreated control plants. Glucanase activity was always reduced by silicon treatment with respect to the untreated control. The inoculation with *Fom* caused an increase of glucanase activity, contrarily to *Pal*, which was never able to induce an enhancement of enzymatic activity (Fig. 1A). Treatments carried out in 2005, did not produce any persistent increase in glucanase activity as resulted in January 2006 analysis (Fig. 1B). Treatments combined with *Pal* inoculation did not produce effects, except for Rootshield, which caused an increase of glucanase activity in July (Fig. 1B). Fig. 1C shows that the combination treatments with *Fom* inoculation increased the glucanase activity, except for silicon in April. It should be noted that, in June assessment, the treatment with Rootshield enhanced particularly the effect of the pathogen *Fom*, which was very slight by itself. All single treatments and inoculations on plants generally produced an increase in chitinase activity; Rootshield showed the highest enzymatic activity in June assessment (Fig. 2A).

Treatments associated with *Pal* inoculation gave a further increase of chitinase activity, with respect to single treatments, in Rootshield+*Pal* (April) and silicon+*Pal* (June). This enhancement was produced mainly by an interaction between treatments and pathogen (Fig. 2B). Values of chitinase activity were overall higher in combinations biological treatment+*Fom* (Fig. 2C) compared to biological treatment+*Pal* as shown in Fig. 2B; in
Rootshield+Fom combination assessed in June, this activity was higher than the single effects of Rootshield and Fom (Fig. 2C).

The video analysis of roots revealed an enhanced apparatus in treated plants: Ekoprop, and to a lesser extent Rootshield, induced abundant hairy roots, while silicon seems to stimulate the root apparatus, but not hairy roots (data not shown).

Figure 1. Time course of glucanase activity in kiwifruit leaves assessed in 2006 after each treatment alone (A), and in combination with Pal (B) and Fom (C). Bars represent standard deviations. The enzymatic activity has been determined as optical density. Pal: P. aleophilum; Fom: F. mediterranea. Time of treatments: May and July 2005 and April and June 2006. Time of inoculation: February 2006.

In 2005, all treatments increased shoot dry weight as compared to control, whereas silicon caused a slightly decrease in the following year. In general, the chlorophyll content was not influenced by biological treatments, while was slightly reduced by silicon application and by inoculation of treated plants with Fom (data not shown).

In conclusion, Ekoprop, Rootshield and silicon treatments caused an increase of glucanase and chitinase activities, which are considered markers associated with systemic acquired resistance. This enhancement was due, in some cases, to the combinations between treatments and inoculations and, in others to pathogens or to the treatments. The enhancement of root apparatus caused mainly by biological treatments can play an important role in withstanding stress (Harman, 2004) especially caused by chronic disease caused by Fom and Pal. Final conclusions will depend on ongoing research to assess the effectiveness of treatments towards the development of the disease caused by artificial infection into the wood.
Figure 2. Time course of chitinase activity in kiwifruit leaves assessed in 2006 after each treatment alone (A), and in combination with Pal (B) and Fom (C). Bars represent standard deviations. The enzymatic activity has been determined as optical density. Pal: *P. aleophilum*; Fom: *F. mediterranea*. Time of treatments: May and July 2005 and April and June 2006. Time of inoculation: February 2006

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**References**


Biocontrol as an alternative for leaf rust management in organically-grown coffee

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Abstract: Alternatives to chemicals are required to control leaf rust on organic coffee (OC) plantations. Thus we started a research program aiming at rust biocontrol. From 393 bacteria and fungi isolated from leaves, debris, and soil from OC farms, 17 isolates reduced both infection frequency and number of spores produced/leaf by more than 70%. Seven isolates (six Bacillus sp. and one Pseudomonas sp.) were the most efficient in reducing rust severity on ‘Catuaí’ plants, mainly when applied before Hemileia vastatrix inoculation. These seven isolates were evaluated in a field experiment set in an OC farm in 2005 (E1) and 2006 (E2). Nine treatments (the bacterial isolates, copper hydroxide, and water) were applied via 3 (E1) or 4 monthly sprays (E2) and rust incidence (RI) was assessed monthly. At E1, sprays started in January, and no treatment reduced rust progress. In November 2005, RI reached 5%, and treatment sprays for E2 started. Treatments differed (P<0.0001) regarding RI in June, increment in RI from December 2005 to June 2006, and area under disease progress curve. Lowest values of the three variables (P<0.0001) were recorded in plots treated with copper hydroxide, one isolate of Bacillus sp. and Pseudomonas sp.

Keywords: antagonists, Bacillus, Hemileia vastatrix, Pseudomonas

Introduction

Worldwide, coffee (Coffea arabica L.) is a high business turnover and Brazil is the largest producing country. Lately, the market-share for high-quality coffee, such as the OC, has remarkably increased. However intensity of diseases is expected to be high in organic production systems, due to restrictions in pesticide usage. Currently, control of leaf rust (Hemileia vastatrix Berk. & Br.), the most damaging coffee disease, is heavily based on fungicides (Zambolim et al., 1997). As rust-durable resistance is difficult to attain and fungicides are restricted in OC, effective alternatives for rust management are needed.

Biocontrol is an interesting option for organic production systems (Harman, 2000). There are promising results in biocontrolling coffee rust with Bacillus subtilis (Bettiol et al., 1994) and with hyperparasite fungi (Gonzalez & Martinez, 1996). No studies were oriented yet to prospect for microorganisms to be used in OC production system. We hypothesized that naturally occurring microorganisms in OC crops are effective in reducing leaf rust intensity. Thus we isolated microorganisms from organically grown coffee plants and tested them as biocontrol agents to H. vastatrix.

Materials and methods

Samples of coffee leaves (diseased, rust-free, or dead) and soil underneath plant canopy were collected in OC plantations in Minas Gerais State, Brazil. Green or dead leaf discs, washate from discs and soil suspensions were plated following different procedures and at different culture media. All colonies formed were stored in PDA in culture tubes at 10°C.
Suspensions of each fungal (10^6 conidia/ml) or bacterial (O.D. 540=0.2) isolate were sprayed on the underside of leaves of 6-month old 'Catuaí' plants. After 48h in a moist chamber at 25°C *H. vastatrix* was inoculated; plants remained 48h in moist chamber at 25°C in the dark, and were taken to a growth chamber at 22°C, 12h day length. Four epidemiological components were assessed: latent period (LP), infection frequency (IF), number of spores produced per leaf (UPL), and spore germination (UG). For all components, treatment efficacy (TE) was estimated in relation to control plants sprayed with water and inoculated with *H. vastatrix*. The screened microorganisms were applied on leaves at 0, 4, 8, 12, or 16 days either before or after *H. vastatrix* inoculation. TE in reducing IF and UPL was estimated as before.

Seven bacterial isolates were selected and evaluated in a field experiment set in an OC farm in a randomized block design with four replicates, in 2005 (E1) and 2006 (E2). Nine treatments were compared: each bacterial isolate (cells diluted to DO 540=0.2), copper hydroxide (0.88 g/plant), and water. Treatments were applied (0.4l/plant) in 3 (E1) or 4 monthly sprays (E2), starting January (E1) and November 2005 (E2). Rust incidence (RI) in 60 leaves/replicate was assessed monthly, from January/2005 to June/2006. Based on RI values from January to May/2005 (E1) and from December/2005 to June/2006 (E2), maximum RI, average increment, and area under disease progress curve (AUDPC) were estimated. Experimental data were analyzed with the SAS System 9.1.

**Results**

A total of 154 bacteria (b) and 239 fungi (f) were isolated: 46 b and 73 f from leaves (washing and discs), 38 b and 69 f from debris, and 70 b and 97 f from soil. No isolate efficiently reduced LP or UG. Treatment efficacy in reducing IF and UPL was more than 70% for 19 isolates. Two fungi that did not sporulate after preservation were discarded. Further experiments were conducted with eight bacterial and nine fungal isolates. Antagonists reduced IF and UPL. Higher efficacy of the 17 isolates was achieved when they were applied before *H. vastatrix* inoculation (Table 1). Antagonist efficacy in reducing IF and UPL varied according application times ($P<0.0001$) and decreased over time.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Efficacy in reducing IF (%)</th>
<th>Efficacy in reducing UPL (%)</th>
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<tbody>
<tr>
<td>CS*2</td>
<td>99a, 98a, 94a, 93a, 92a</td>
<td>100a, 99a, 99a, 97a, 97a</td>
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<tr>
<td>B157*4</td>
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<td>100a, 92ab, 96a, 90ab, 84a</td>
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<tr>
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<td>100a, 99a, 97a, 83ab, 81a</td>
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<td>98a, 89ab, 95a, 83ab, 88a</td>
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<tr>
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<td>97a, 98ab, 94a, 79ab, 83a</td>
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<td>B205</td>
<td>96a, 79a, 81a, 60a, 58a</td>
<td>99a, 89ab, 85a, 52b, 39b</td>
</tr>
</tbody>
</table>

*Days before inoculation; *2Copper sulfate; *3means with the same letter/column are not different (Tukey test, $\alpha=0.05$); *4B or P= isolates of *Bacillus* or *Pseudomonas*, respectively.
Under field conditions, when the sprays started in E1 (January 2005), RI was above 15%. Trends of disease progress were similar for all treatments: RI increased, reached a peak in May, and decreased further. No significant effects of isolates were found, but treatments tended to reduce RI in May, RI increment from January-May, and AUDPC values (Table 2). After June, RI values decreased. In November, all treatments had similar progress curves and RI reached 5% (a threshold value to trigger chemical control), and treatment sprays for E2 started. The treatments did not differ \((P=0.72)\) regarding RI in December, but differed \((P<0.0001)\) regarding RI in June 2006, increment in RI from December-June, and AUDPC (Table 2). In general, lower values of the three variables were recorded in plots treated with copper hydroxide, one isolate of Bacillus sp., and the isolate of Pseudomonas sp.

Table 2. Initial incidence (I), maximum incidence (M), average increment (M-I), and area under disease progress curve (AUDPC) of leaf rust in organically-grown coffee plants sprayed with different treatments (Treat), at two experimental runs (E1 and E2)

<table>
<thead>
<tr>
<th>Treat</th>
<th>I</th>
<th>M</th>
<th>M-I</th>
<th>AUDPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1</td>
<td>E2</td>
<td>E1</td>
<td>E2</td>
</tr>
<tr>
<td>CuH*1</td>
<td>0.19a</td>
<td>0.07a</td>
<td>0.46a</td>
<td>0.21c</td>
</tr>
<tr>
<td>B157*3</td>
<td>0.24a</td>
<td>0.05a</td>
<td>0.48a</td>
<td>0.28c</td>
</tr>
<tr>
<td>P286</td>
<td>0.27a</td>
<td>0.06a</td>
<td>0.37a</td>
<td>0.39b</td>
</tr>
<tr>
<td>B25</td>
<td>0.25a</td>
<td>0.06a</td>
<td>0.52a</td>
<td>0.57a</td>
</tr>
<tr>
<td>B10</td>
<td>0.28a</td>
<td>0.06a</td>
<td>0.46a</td>
<td>0.50a</td>
</tr>
<tr>
<td>B281</td>
<td>0.22a</td>
<td>0.06a</td>
<td>0.38a</td>
<td>0.53a</td>
</tr>
<tr>
<td>B175</td>
<td>0.31a</td>
<td>0.05a</td>
<td>0.41a</td>
<td>0.50a</td>
</tr>
<tr>
<td>B205</td>
<td>0.24a</td>
<td>0.06a</td>
<td>0.40a</td>
<td>0.54a</td>
</tr>
<tr>
<td>Water</td>
<td>0.20a</td>
<td>0.07a</td>
<td>0.64a</td>
<td>0.53a</td>
</tr>
</tbody>
</table>

*1Copper hydroxide; *2means with the same letter/column are not different (Tukey test, \(\alpha=0.05\)); *3B or P= isolates of Bacillus or Pseudomonas, respectively.

Discussion

Efficient antagonists to *H. vastatrix* were isolated from the different places and materials sampled, as reported previously (Sultana et al., 2000; Yuen et al., 2001). In our search, the potential antagonists to *H. vastatrix* came from OC, which probably would confer them advantages in establishing in coffee plants. Six selected bacteria are Bacillus spp. and one is a Pseudomonas sp. As species of both genera can have different antagonistic mechanisms (Bettiol et al., 1994; Porras et al., 1999; Enebak & Carey, 2000; Raaijmakers et al., 2002; Teixeira et al., 2005), the ones we selected were considered as potential biocontrol agents.

Application of the seven isolates protected coffee plants against *H. vastatrix* for at least 16 days. A commercial product of *B. thuringiensis* promoted systemic protection of coffee plants against *H. vastatrix* (Roveratti et al., 1989), supposedly by inducing resistance (Cristancho & Leguizamón, 1995). However, at the moment, no claim can be made regarding the mechanisms of action of the bacteria under evaluation.

Fungicide sprays to control rust usually start by November (beginning of rainy season), when RI ≤5% (Zambolim et al., 1997). At E1, the sprays began in January, and RI was above the threshold. Thus no treatment, even fungicide, reduced RI. However, maximum RI in May, RI increment from January-May, and AUDPC values were larger in the control plots.
Coffee crops are perennial and years of high and low yields alternate. As this fact makes experimental results gathered in a single year questionable, field experiments continued. At E2, two bacterial isolates were as efficient as copper fungicide in keeping disease intensity at low levels. Another experimental run is projected for 2006/2007.

It is believed that natural biological control is favored under organic systems, so introduced biocontrol agents also are likely to succeed (Harman, 2000). Rust biocontrol is expected to become a reality for both conventional and organically coffee production systems. In OC, there are restrictions to fungicide usage, whereas in conventional coffee reduction of excessive fungicide is needed. Complementary studies are being conducted to better support the implementation of rust biocontrol.

Acknowledgements

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References

Biocontrol agents
Modes of action
Population-level evidence of the importance of 2,4-diacetylphloroglucinol and hydrogen cyanide in plant protection by *Pseudomonas fluorescens*

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**Abstract:** Mutant analysis has shown that hydrogen cyanide and 2,4-diacetylphloroglucinol contribute to biocontrol by pseudomonads, but for 2,4-diacetylphloroglucinol this was not confirmed by population studies of biocontrol strains. Here, *in planta* comparison of 230 biocontrol pseudomonads from a screening of 3132 bacterial isolates obtained from 63 soils world-wide showed that cyanide and especially 2,4-diacetylphloroglucinol were indeed associated with superior biocontrol in the *Pythium ultimum*-cucumber and *Fusarium oxysporum*-tomato pathosystems.

**Introduction**

Many biocontrol fluorescent pseudomonads protect plants from soil-borne diseases by the production of antimicrobial secondary metabolites including 2,4-diacetylphloroglucinol (Phl) and hydrogen cyanide (HCN) (Haas & Défago, 2005). The importance of Phl and HCN in biocontrol was evidenced for a few strains in experiments where wild-type pseudomonads protected better than mutant derivatives in which production of HCN (Voisard et al., 1989) or Phl (Keel et al., 1992) was impaired. Population studies of root-colonizing Pseudomonads in disease-suppressive and conducive soils also suggested that Phl⁺ and/or HCN⁺ strains played a role in biocontrol, but these were also found in conducive soils (Raaijmakers et al., 1997). Since most plant growth promoting (PGPR) pseudomonads produce more than one biocontrol metabolite and genetic inactivation of one trait can modify the expression of other biocontrol traits (Haas & Défago, 2005), it is difficult to assess the relative importance of each compound.

The aim of the current work was to perform a statistically-unbiased comparison study of wild-type biocontrol pseudomonads to confirm at population level that the ability to produce HCN and/or Phl is indeed associated with superior plant protection. In order to be statistically meaningful, this assessment was done using a much larger collection of biocontrol pseudomonads compared with previous studies, and the biocontrol pseudomonads were obtained following an homogenous *in planta* screening protocol starting from 3132 bacterial isolates collected in six consecutive years from roots of plants grown in different soils of worldwide origin.
Methods and results

Bacterial isolation

Topsoil (upper layer 0-20 cm) was collected at 63 locations in 19 countries representing every arable continent, including disease-suppressive soils from Switzerland, Italy and Ghana. For each soil sample, one pot was sown with surface-disinfected seeds of each of the following bait plants, which served as an enrichment for rhizosphere-competent bacteria: cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*), wheat (*Triticum aestivum*), tobacco (*Nicotiana glutinosa*), cotton (*Gossypium hirsutum*), bean (*Phaseolus vulgaris*) and radish (*Raphanus sativus*). After 2-3 weeks the plants were harvested and singular root-associated bacterial colonies were collected by dilution plating on the semi-selective *Pseudomonas* media S1 and King’s B, yielding a total of 3132 isolates.

Identification of biocontrol isolates in the *Pythium ultimum*-cucumber pathosystem

All bacterial isolates were screened for biocontrol activity either *in vitro* by measuring *P. ultimum* inhibition on KB and malt agar, or *in planta* by assessing biocontrol of *Pythium* damping-off of cucumber, as described by Sharifi-Tehrani et al. (1998). The biomass of emerged plants was recorded. Biocontrol activity (\%) was calculated as \(\{1 - [(W_C - W_I) / (W_C - W_P)]\} \times 100\), using plant fresh weight obtained in presence of the isolate tested (W_i), in the control with no microorganisms added (W_C) and in the unprotected control with the pathogen alone (W_P). Over the 6-year survey, a total of 307 biocontrol bacteria were thus selected based on at least 5-mm inhibition zone on KB or malt agar (*in vitro* experiments) or significant plant protection, especially in comparison with the biocontrol performance of the model biocontrol strain CHA0 (*in planta* experiments). Among the 307 biocontrol isolates selected, 230 were identified as fluorescent pseudomonads based on the abilities to grow on S1 plates and to display fluorescence under UV light (366 nm) on succinate minimal medium supplemented with 100 µg ml\(^{-1}\) EDDHA. They originated from cucumber, tomato, wheat, tobacco, cotton, bean and also from bulk soil (Table 1). Evaluation by restriction analysis of 16S rDNA (Keel et al., 1996) or 16S-23S rDNA amplicons (Sharifi-Tehrani et al., 1998), catabolic profiling (Wang et al., 2001), and diversity analysis of *phlD* (Wang et al., 2001), *hcnBC* (Ramette et al., 2003b), *hrcN* and *rrs* (Rezzonico et al., 2004), indicated that most/if not all of our isolates were unique strains, even those originating from the same field site. This ensured that we considered a genetically diverse group of strains in our correlation study.

Table 1. Characterization of isolates from different bait plants based on their biocontrol activity and the production of the secondary metabolites HCN and Phl

<table>
<thead>
<tr>
<th>Bait plant</th>
<th>Isolates</th>
<th>Cyanide producers</th>
<th>Phl producers</th>
<th>Ratio</th>
<th><em>P. ultimum</em>-cucumber biocontrol %</th>
<th>FORL-tomato biocontrol %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HCl%</td>
<td>%HCl’</td>
<td>Phl%</td>
<td>%Phl’</td>
<td>Phl/HCl</td>
</tr>
<tr>
<td>Cucumber</td>
<td>110</td>
<td>79</td>
<td>71.8</td>
<td>23</td>
<td>20.9</td>
<td>0.92</td>
</tr>
<tr>
<td>Tomato</td>
<td>43</td>
<td>39</td>
<td>90.7</td>
<td>27</td>
<td>62.8</td>
<td>0.69</td>
</tr>
<tr>
<td>Wheat</td>
<td>42</td>
<td>23</td>
<td>54.8</td>
<td>9</td>
<td>21.4</td>
<td>0.39</td>
</tr>
<tr>
<td>Tobacco</td>
<td>25</td>
<td>17</td>
<td>68.0</td>
<td>14</td>
<td>56.0</td>
<td>0.62</td>
</tr>
<tr>
<td>Bean</td>
<td>4</td>
<td>3</td>
<td>75.0</td>
<td>1</td>
<td>25.0</td>
<td>0.33</td>
</tr>
<tr>
<td>Cotton</td>
<td>2</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Soil</td>
<td>2</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>230</strong></td>
<td><strong>165</strong></td>
<td><strong>74.7</strong></td>
<td><strong>76</strong></td>
<td><strong>33.0</strong></td>
<td><strong>6.46</strong></td>
</tr>
</tbody>
</table>
Analysis of biocontrol pseudomonads in two pathosystems

The 230 biocontrol pseudomonads were further tested for biocontrol activity in the *P. ultimum*-cucumber pathosystem described above and also in the *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL)-tomato pathosystem: each isolate was studied using three rockwool cubes (with 12 tomato seedlings each) soaked in plant nutrient solution containing $10^6$ conidia of FORL and $10^7$ CFU of the pseudomonad per ml. After 14 days of growth, disease severity was scored and biocontrol activity (%) computed as $\{1 - \frac{(I_c - I_1)}{(I_c - I_P)}\} \times 100$, in which $I$ values represent disease indices (0: healthy seedlings; 1: one or two brown marks on the roots or the crown; 2: several small brown marks or a few large lesions; 3: extensive root necrosis but plant still alive; 4: plant dead). Results indicated that a majority of *Pseudomonas* isolates had biocontrol potential in both pathosystems, i.e. reaching thresholds of at least 30% and 20% biocontrol level in the *P. ultimum*-cucumber and FORL-tomato pathosystems, respectively (Fig. 1). Cucumber isolates represented almost half the biocontrol pseudomonads identified after initial screening in the cucumber-based system (Table 1), but collectively they did not display higher biocontrol ability in comparison with the non-cucumber isolates in the *P. ultimum*-cucumber pathosystem.

Relationship between biocontrol and the ability to produce Phl

The ability of biocontrol fluorescent pseudomonads to produce Phl was determined by extraction from 3-day-old liquid KB cultures with 80% acetone followed with HPLC analysis, as described (Keel et al., 1996). Phl production ability was found in 76 of the 230 biocontrol fluorescent pseudomonads (i.e. 33%). In each pathosystem (Fig. 1A-B), it was associated with superior disease suppression ability, as indicated by the fact that as many as 26.3% of Phl⁺ isolates exceeded a 60% biocontrol level in the *P. ultimum*-cucumber pathosystem (vs. 1.3% of Phl⁻ isolates), whereas only 1.3% were below a 30% biocontrol level (vs. 58.4% of Phl⁻ isolates). A similar situation was observed in the FORL-tomato system. All Phl⁺ *Pseudomonas* strains published so far but one have been found to also produce HCN, complicating efforts to determine the relative importance of each biocontrol compound in plant protection. When only the subset of HCN⁺ isolates was considered, Phl production ability was again associated with superior biocontrol performance (Fig. 1G-H) regardless of the pathosystem studied. When comparing the data from the different bait plants a good correlation between the percentage of Phl⁺ strains isolated and their overall average biocontrol activity was observed (Table 1), especially in the *P. ultimum*-cucumber system ($R^2=0.94$). This may explain the relative poor performance of cucumber isolates (low proportion of Phl producers) and the good performance of tomato isolates (high proportion of Phl producers). The correlation with biocontrol was lower when the proportion of HCN⁺ strains was considered. These findings on the superiority of Phl⁺ biocontrol pseudomonads over Phl⁻ biocontrol pseudomonads are important, because despite the demonstration of the role of Phl in a few individual strains and the quantitative prevalence of Phl⁺ pseudomonads in suppressive soils, this superiority has failed so far to be demonstrated when statistically comparing a range of Phl⁺ and Phl⁻ wild-type biocontrol strains (Sharifi-Tehrani et al., 1998; Ellis et al., 2000).

Relationship between biocontrol and the ability to produce HCN

Production of HCN was studied qualitatively on KB plates using the indicator paper described by Castric & Castric (1983) and was found in as many as 165 of the 230 biocontrol fluorescent pseudomonads selected. When the whole collection was analyzed, HCN production ability was associated with effective disease suppression ability in each pathosystem (Fig. 1C-D).
Figure 1. Distribution of *Pseudomonas* isolates with respect to their biocontrol activity in the *P. ultimum*-cucumber (A, C, E, G) and FORL-tomato (B, D, F, H) pathosystems. Producers (black bars) of antimicrobial secondary metabolites Phl (A, B) and HCN (C, D) were compared with the respective non-producers (white bars) in their ability to protect in each particular pathosystem. The effect of HCN production ability in Phl non-producers is depicted in E and F, and that of Phl production ability in HCN producers in G and H. The biocontrol activity was computed based on plant fresh weight (*P. ultimum*-cucumber) or disease index (FORL-tomato) and the asterisk indicates the position of *Pseudomonas* strain CHA0 used as reference biocontrol agent over the six years of the study (41.2% average biocontrol activity in the *P. ultimum*-cucumber pathosystem and 29.6% average biocontrol activity in the FORL-tomato pathosystem). The *P* values at which the two distributions are different in a Kruskal-Wallis one-way analysis are shown within each graph.

When only Phl+ strains were considered, it appears that higher biocontrol responses were again obtained with HCN+ strains compared with HCN- strains in the FORL-tomato pathosystem (Fig. 1F). However, a different situation was found in the *P. ultimum*-cucumber pathosystem, where HCN production ability gave less significant results (Fig. 1E). These results, which are in agreement with data obtained from mutant analysis (Voisard et al., 1989), strengthen previous findings from the assessment of strain collections (Sharifi-Tehrani et al., 1998; Ramette et al., 2003a) on the importance of HCN production ability for superior biocontrol. However, they also suggest that the ability to produce HCN is statistically of less significance for biocontrol than that of Phl, in contrast to findings obtained with a smaller strain collection in another *P. ultimum*-base pathosystem (Ellis et al., 2000). It is important to note that all 76 Phl+ isolates but one also produced HCN. The amplicon obtained from the sole Phl+HCN- strain using primers for *hcnBC* (Ramette et al., 2003a) was smaller than the 587-bp fragment amplified in HCN+ *Pseudomonas* strains. Sequencing revealed a 134-bp deletion in the N-terminal domain of *hcnC*, corresponding to positions 2024-2157 of the *hcnBC* sequence of strain CHA0 (accession number AF053760). Hence, extrapolation of the
data from our large collection strongly suggests that Phl production may be restricted to genotypically HCN⁺ strains.

**Discussion**

Here, we focused on Phl because it is involved in biocontrol of a broad spectrum of diseases by many antagonistic strains (Haas & Défago, 2005), and this is the first unequivocal population-level evidence that pseudomonads producing Phl can protect plants better than non-producing biocontrol pseudomonads. Population statistics demonstrates that also HCN producing ability is a positive attribute for plant-associated biocontrol *Pseudomonas* strains, especially when linked to the ability to also produce Phl. The best strains in each system weren’t necessarily as effective in the other system, demonstrating that the host plant species still has a significant influence on the dynamics, composition and activity of specific indigenous antagonistic *Pseudomonas* spp. Nevertheless, a weak positive but significant correlation was found between the biocontrol levels in the two pathosystems (r=0.48; n=164, P<0.01). This demonstrates that some bacterial features, i.e. Phl and HCN production, are indeed broad-spectrum positive traits for biocontrol.

When it comes to finding new biocontrol agents, effective strategies to replace the labor-intensive *in planta* screenings are lacking. Our results suggest that Phl and HCN could be used in combination as biocontrol markers for streamlining this process. PCR protocols are available to identify *phl* (McSpadden Gardener et al., 2001) and *hcn* genes in *Pseudomonas* (Ramette et al., 2003a). Since Phl production is found naturally only in HCN producers, and because HCN is much simpler to detect, the search for additional Phl⁺ strains may be preceded by a rapid phenotypic screen for HCN⁺ isolates.

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**References**


Molecular strategies to study different mode of action of rhizobacterial strains with biocontrol activity in the *Rosellinia/avocado* test system

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Abstract: Avocado (*Persea americana* L.) is a crop recently introduced in Southern Europe with promising economic perspectives. The most destructive disease of this crop in the Mediterranean area is white root rot, caused by the fungus *Rosellinia necatrix*. Control of white root rot is complex, and recently, different microorganisms with potential biocontrol activity have been isolated and identified in order to improve in the future the integrated management of this crop. To improve knowledge on the mode of action of these biocontrol microorganisms, different strategies were carried out, mainly based in the construction of mutants.

A first strategy was studied on the antagonistic rhizobacterial strain *Pseudomonas fluorescens* PCL1606, which only produced the antibiotic 2-hexyl, 5-propyl resorcinol (HPR). In order to study the role of HPR in biocontrol, two approaches of mutagenesis were carried out. The first approach was the construction of derivative strains impaired in the antagonistic activity by *Tn5* mutagenesis. The *Tn5* flanking regions were analyzed, revealing putative genes involved in the production of HPR. A second approach was carried out by site-directed mutagenesis in an operon previously described in *P. aurantiaca*, and involved in the HPR biosynthesis (*dar* operon). Some *dar* genes were detected in *P. fluorescens* PCL1606. Site-directed mutants on these genes in *P. fluorescens* PCL1606 showed reduction in the antagonistic activity against different fungi. In both types of mutants, production of HPR was assessed, and biocontrol experiments on experimental test systems were carried out, showing reduction of the protection levels when using these derivative mutants.

A second strategy was based on rhizobacterial strains with colonization traits. Several bacterial strains were isolated from the roots of symptomless avocado trees, and some of them could prevent in vitro growth of *R. necatrix*. Colonization of avocado roots by these strains was tested in vivo and, two strains of *P. alcaligenes* (GBF.1.11 and GBF.2.18) were selected by their high colonization efficiency. None of these strains produced detectable antifungal antibiotics. Biocontrol assays against *R. necatrix* showed that while GBF.2.18 could prevent fungal growth, GBF.1.11 did not. To reveal the importance of the colonization in the biological control process, two different genetic approaches were used to enhance root tip colonization on these isolates by: a) direct selection from a mini-*Tn5* pool of mutants; b) knock-out of *mutY* in *Pseudomonas* spp. Loss of this function in a *P. fluorescens* strain has been reported to increase the number of spontaneous mutations. Inoculation of a *mutY* mutant into the root system of tomato selected for strains showing enhanced competitive root tip colonization.
Mode of action of *Bacillus subtilis* as biocontrol agent of fruit diseases

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**Abstract:** South Africa is a major fresh fruit exporting country to the European community. This requires that export fruit must adhere to international quality and safety standards and comply with European Union and retailer maximum residue limits. Due to the lack, or prohibited use, of effective fungicides to protect fresh produce during extensive shipping and distribution systems, as well as stringent MRL requirements, producers are more reliant on safe biocontrol alternatives. Although biological control products can provide effective alternative means of controlling disease, consistent product performance remains a major challenge and requires a deeper understanding of how biocontrol- and pathosystems function over time and space. This study focuses on the different modes of action of one commercial biocontrol agent, *Bacillus subtilis* (Avogreen®), registered for control of avocado pre- and postharvest diseases, i.e. Cercospora spot and anthracnose, and compares it with similar potential biocontrol agents of citrus (*B. subtilis* and *B. licheniformis*), mango (*B. licheniformis*) and the antagonistic yeasts *Candida sake* and *Cryptococcus laurentii* on citrus. Mode of action studies included *in vitro* dual culture methods, volatile assessment studies, production of siderophores and secondary compounds, and direct interaction assessed with electron microscopy. The antagonist *B. subtilis* directly interacted with *Colletotrichum gloeosporioides* on avocado fruit. Diffusible inhibitory metabolites, volatiles, siderophores and enzymes were produced *in vitro*. The other *Bacillus* antagonists on mango and citrus were mainly reliant on antibiosis, competition and volatile production. *Bacillus* spp. relied on several modes of action, whereas the yeasts were predominantly competitive colonizers.

**Key words:** biofungicide, post-harvest pathogens, subtropical fruit

**Introduction**

The growing global demand for fresh tropical and subtropical fruit of high quality and without pesticide residues necessitates the development of alternative disease control approaches. Due to their highly perishable nature, avocado, *Persea americana* Miller and mango, *Mangifera indica* L. are subject to various pre- and postharvest fruit diseases. In both crops, classic preharvest quiescent infections by *Colletotrichum gloeosporioides* Penz. (Penz. and Sacc.) are difficult to detect at harvest and can subsequently cause major post-harvest losses on the export market. In the case of citrus (*Citrus sinensis* L.), postharvest losses are mainly caused by the wound pathogen *Penicillium digitatum* (Pers.) Sacc., which can infect during picking, packing and handling and manifest at the retail end. These pathogens are currently effectively controlled with postharvest fungicides. However, with the new pesticide legislation and stricter maximum residue levels (MRL’s) set by the European Union (EU), exporting countries, particularly in the southern hemisphere, are more reliant on effective protection against postharvest decay.

Due to the extended export chain, caused by lengthy shipment, fruit need to be protected, forcing growers to evaluate alternative disease control options. The commercial
product, Avogreen® (*B. subtilis*), originally isolated from the avocado fructoplane (Korsten et al., 1995), is commercially sold in South Africa as a preharvest spray- or postharvest dip application registered for control of Cercospora spot and anthracnose respectively on avocado (Korsten, 2004, 2006). Although the product has shown effective control over several seasons, optimal product performance under various environmental conditions remains a challenge. Even in successful biocontrol strategies, a more in depth understanding of the mechanism of action may improve the reliability of the agent as well as broadening its commercial application to other pathosystems.

The modes of action of *B. subtilis* against *C. gloeosporioides* on avocado (Demoz & Korsten, 2006) and *P. digitatum* on citrus (Obagwu & Korsten, 2003) have been investigated at Plant Pathology Laboratories (University of Pretoria, South Africa). Similar studies have been conducted using *B. licheniformis* to control anthracnose on mango (Govender et al., 2005) and yeasts to control postharvest decay on citrus (unpublished data). In this study, various modes of action have been considered in order to obtain a better understanding of evolutionary forces driving antagonist-pathogen-host interactions.

### Material and methods

**Isolates**

Subcultures of *B. subtilis* MI-14 (Avogreen®), F1 (Citrigreen), *B. licheniformis* (Mangogreen), *Candida sake* and *Cryptococcus laurentii* were used in this study. Fresh cultures were maintained on Standard 1 (STD 1) nutrient agar (Biolab, Johannesburg) or Potato Dextrose Agar (PDA) (Biolab). The plates were incubated at 28±3°C for 24-48 h. A loopful of each pure culture was transferred to a 250 ml conical flask, each containing 50 ml Nutrient Broth (Biolab) or Malt Extract Broth (Biolab), and incubated overnight on a rotary shaker (150 rpm) at 25°C. Cultures were centrifuged for 15 min. at 750 g using a Labofuge GL centrifuge. The resulting pellets were washed and re-suspended in sterile quarter-strength Ringer’s (Merck) solution. Cell counts were performed with a Petroff-Hausser counting chamber. The cell concentration used in the experiments was standardized to 10^7 cfu ml⁻¹.

Mycelium-containing agar plugs (5 mm diameter) of the fungal pathogens (*C. gloeosporioides* and *P. digitatum*) were placed on PDA and incubated at 25-28°C for 3-7 days prior to use. Conidia were subsequently harvested by flooding the surface of the media with sterile distilled water and gentle agitation to dislodge the conidia. The inoculum concentration used was standardized at 10^6 conidia ml⁻¹ as commonly used in citrus post-harvest experiments (Eckert and Ogawa, 1988).

**Competition for space and nutrients**

An amended dual culture method of Skidmore (1976) was used in this study. The methods of Schwyn & Neilands (1987), Buyer et al. (1989) and Alexander & Zuberer (1991) were combined to evaluate siderophore production.

**Host colonization**

Excised avocado, citrus or mango skin samples were prepared and inoculated according to the method described by Dawson et al. (1969). Samples were dried in a Hitachi HCP-2 critical point dryer and mounted on aluminum stubs. Specimen stubs were coated with gold palladium in an Eiko IB-3 ion coater. Stubs were viewed in a Hitachi 840 JEOL SEM operating at 5 kV.

**Volatile production**

The potential production and inhibitory effect of volatiles on *Penicillium* and *Colletotrichum* spp. were assessed using the sealed plate method of Fiddaman & Rossall (1993). Measure-
ments of fungal radial growth were taken over a 3-day period with inoculated plates incubated at 25°C.

**Enzyme production**
The *in vitro* production of chitinase, amylase, lipase and proteinase by the antagonists and their inhibitory effect on *Penicillium* and *Colletotrichum* spp. were investigated on selective media (Skinner & Lovelock, 1979; Frändberg & Schnürer, 1994).

**Secondary compounds**
A production medium for antifungal compounds (McKeen et al., 1986) was inoculated with the respective antagonists and shake incubated at 25°C for 7 days. Two ml of the suspension was aseptically removed daily and filtered through a 0.22 µm pore sized acetate filter (Millipore, Separation Scientific, Johannesburg) to obtain a cell-free suspension. To determine antifungal activity, conidia suspensions of *C. gloeosporioides* were spread-plated on PDA. Wells were made in the agar with a sterile 5 mm diameter cork borer and 0.25 ml of the bacterial filtrate was pipetted into the wells. The absence or presence of inhibition zones were noted and measured. Sterile medium served as the negative control. All experiments consisted of three replicates and were repeated twice for each pathogen-antagonist combination. Data were analyzed using the statistical program GenStat (2000).

**Results and discussion**
In this study, all the *Bacillus* isolates effectively inhibited *in vitro* pathogen growth, which was evident through the presence and size of the inhibition zones. The presence and fungitoxic activity of volatiles and secondary compounds were also confirmed (data not shown). This is in agreement with the findings of Korsten et al. (1997) that *B. subtilis* effectively controlled pre- and postharvest diseases of avocado, involving several modes of action, which included production of an inhibitory substance. Inoculation of the avocado fruit surface with *B. subtilis* followed by challenge inoculation with the pathogen showed inhibition of *C. gloeosporioides* conidia germination (Fig. 1a). *Bacillus* cells were also observed actively dividing on the fruit surface and colonizing the pathogen with extracellular slime and micro-colonies around the cells (Fig. 1b).

![Figure 1. Scanning electron micrographs of avocado (a) inoculated with *Colletotrichum gloeosporioides* followed by *Bacillus subtilis*, (b) showing antagonist colonization on the fungal hyphae. Abbreviations: C = conidia of *C. gloeosporioides*, B = *B. subtilis* cells, H = hyphae of *C. gloeosporioides*.](image-url)
Similar results were found for the other bacterial antagonist-pathogen combinations. *Bacillus licheniformis* also showed effective control of mango fruit diseases with pre- and postharvest applications (Govender et al., 2005; Silimela & Korsten, 2005). The commercial product, Mangogreen®, originally isolated from the mango phylloplane, is currently in the process of registration in South Africa. In addition, a citrus fructoplane biocontrol agent, Citrigreen®, (*B. subtilis* and *B. licheniformis*), effective against *Penicillium* spp. in citrus for postharvest applications, is also in the registration process (Obagwu & Korsten, 2003). The two yeasts, *C. sake* and *C. laurentii*, isolated from citrus plantations in Ethiopia, also showed promising potential as citrus postharvest dip applications. The predominant mode of action of these agents was competitive colonization (data not shown). Further trials are currently conducted to identify suitable biocontrol agents to control postharvest decay on litchi.

References


Comparative genomics and regulation of cyclic lipopeptide synthesis in antagonistic *Pseudomonas fluorescens*

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**Abstract:** Cyclic lipopeptides are produced by a variety of bacteria, including plant-associated *Pseudomonas* and *Bacillus* species. *Ps. fluorescens* strain R1SS101, isolated from the wheat rhizosphere, has biocontrol activity against a variety of plant pathogens. The activity of strain R1SS101 is determined, in part, by the production of massetolide A, a cyclic lipopeptide surfactant of nine amino acids linked to a 10-C fatty acid. Massetolide A lyses zoospores of oomycete pathogens, including *Phytophthora* and *Pythium* species, and is involved in the attachment-detachment to surfaces and in motility of strain R1SS101. Massetolide A deficient mutants were generated by random mutagenesis and approximately 25 genes involved in massetolide A production were identified. Among these were three large non-ribosomal peptide synthetase (NRPS) genes, designated *massA*, *massB* and *massC*, with a total size of approximately 30-kb. The *massA-C* genes harbor in total nine modules with adenylation, condensation and thiolation domains. The predicted specificity-conferring codes of the nine adenylation domains in MassABC are consistent with the number and composition of the amino acids in the peptide moiety of massetolide A. Despite the colinearity between gene sequence and products, the *massA* gene is disconnected from the *massBC* genes. Furthermore, internal epimerization domains are lacking in the massetolide A synthetic template, suggesting that external racemases are responsible for the L- to D-conversion of the amino acids in the peptide moiety of massetolide A. In addition to these structural genes, a number of regulatory genes involved in massetolide A production were identified, including the *gacA/gacS* two-component regulatory genes. Our current knowledge on the activity, biosynthesis and regulation of cyclic lipopeptide synthesis in antagonistic strains of *Ps. fluorescens* will be presented in detail.
Synergy between phenazines and biosurfactants in the biological control of Pythium induced soil-borne diseases is a general phenomenon in fluorescent pseudomonads

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Abstract: Phenazines and rhamnolipid-biosurfactants produced by Pseudomonas aeruginosa PNA1 act in synergy in the biological control of Pythium spp. In this study, we investigated whether a synergistic interaction between phenazines and biosurfactants occurs in other fluorescent pseudomonads as well. Plant experiments demonstrated that synergy between phenazines and biosurfactants may also occur in other fluorescent pseudomonads such as the WT strains Pseudomonas chlororaphis PCL1391, Pseudomonas CMR5c and CMR12a. Biosurfactant or phenazine mutants of these WT strains lost all biocontrol activity, despite the fact that the mutants still produced one of the antifungal metabolites (phenazines, biosurfactants). Furthermore, we investigated if all phenazine-producing pseudomonads synthesize biosurfactants. A collection of 25 phenazine-producing wild type strains was tested for the production of biosurfactant with the drop collapse technique. Forty percent of all phenazine-producing bacteria had the ability to synthesize biosurfactants. Interestingly, there appeared to be an inherent link between the production of biosurfactants and PCN since all biosurfactant-producing WT strains produced PCN.

Key words: cocoyam, Pythium myriotylum, signal molecule

Introduction

Phenazines and biosurfactants are secondary metabolites commonly produced by fluorescent pseudomonads. Phenazines are heterocyclic, nitrogen-containing brightly colored antibiotics with broad-spectrum activity (Mavrodi et al., 2006). The most commonly identified phenazine derivatives produced by Pseudomonas spp. are pyocyanin, phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), and hydroxy phenazines. Biosurfactants are surfactant-active molecules of biological origin. The production of a specific biosurfactants is generally associated with a specific microbial genus. Rhamnolipids (Ochsner et al., 1994) and cyclic lipopeptides (Raaijmakers et al., 2006) have been identified as important biosurfactant compounds produced by pseudomonads. Although phenazines and biosurfactants have their own physiological role to the producer strain, both metabolites contribute to the ecological competence of the producer.

We have demonstrated that phenazines and rhamnolipids produced by Pseudomonas aeruginosa PNA1 act in synergy in the biological control of soil-borne diseases caused by Pythium spp. (Perneel et al., submitted). Plant experiments showed that the production of phenazines or biosurfactants alone did not suffice for an optimal biological control activity. It is hypothesized that biosurfactants facilitate the access of phenazines into the host cell through the formation of ion channels in the host membrane. One may wonder if root-associated Pseudomonas bacteria always invest in the production of both phenazines and biosurfactants in order to assure their survival on plant roots, since the production of one...
metabolite is not sufficient. Therefore, we examined in this study whether all phenazine-producing *Pseudomonas* spp. synthesize biosurfactants. Furthermore, we verified if synergy between phenazines and biosurfactants is a general phenomenon in the biocontrol of soil-borne *Pythium* spp. by fluorescent pseudomonads.

**Material and methods**

*Microorganisms and culture conditions*

*Pseudomonas* strains CMR5a, CMR5b, CMR5c, CMR12a, CMR12b, CMRama14, and CMRAma 20 were isolated from the rhizosphere of cocoyam in Cameroon (Perneel, 2006). *Pseudomonas* PHZH13, PHZH19, PHZH22, PHZH23, PHZH24, PHZH26, PHZH44, and PHZH 48 were isolated from the tomato rhizosphere in Dijon (France) and were kindly supplied by Jos Raaijmakers from Wageningen University. The *Pseudomonas* strains W15apr36-2, W15jun30, W2apr9, W2aug1, W2aug2a, W2aug3, and W2aug5 originated from the Woluwe river in Brussels and were kindly given by Sandra Matthijs from Vrije Universiteit Brussel (Belgium). Furthermore, three *Pseudomonas chlororaphis* strains were included in the study: *P. chlororaphis* PCL1391 (Chin-A-Woeng et al., 1998) and *P. chlororaphis* LMG1245 and LMG5004. To study the synergistic interaction between phenazines and biosurfactants *in vivo*, PCL1119 - a phenazine mutant of PCL1391 - and CMR5c 9.47 and CMR12a 7.97, two biosurfactant mutants of *Pseudomonas* CMR5c and CMR12a respectively, were used in plant experiments. *Pseudomonas* strains were routinely grown on King’s B (KB)-medium and were incubated at 28°C. The phytopathogen *Pythium myriotylum* CM1, isolated from diseased cocoyam roots in Cameroon (Perneel et al., 2006), was grown on potato dextrose agar (PDA) at 28°C.

*Screening of phenazine-producing Pseudomonas WT strains for biosurfactant production*

A collection of 25 phenazine-producing *Pseudomonas* wild type (WT) strains was screened for the production of biosurfactants using the drop collapse technique. Briefly, cells from a single colony were transferred with a sterile toothpick to a droplet of 50 µl sterile water on parafilm. Production of biosurfactants was confirmed when the droplet collapsed.

*Synergistic interaction between phenazines and biosurfactants in the biocontrol of Pythium myriotylum in different fluorescent pseudomonads*

Plant experiments were carried out to verify if the synergistic interaction between phenazines and biosurfactants in the biocontrol of soil-borne *Pythium* spp. is a common phenomenon in fluorescent pseudomonads. Phenazine mutants of *Pseudomonas chlororaphis* PCL1391 and biosurfactant mutants of *Pseudomonas* CMR5c and CMR12a were tested for disease suppression against *Pythium myriotylum* on cocoyam. Bacteria were inoculated in sterilized volcanic soil collected from Cameroon. Bacteria were inoculated at a concentration of $3 \times 10^6$ CFU g$^{-1}$ soil, 48 h before inoculating the soil with *P. myriotylum* (1250 propagules g$^{-1}$ soil). Tissue derived cocoyam plantlets were transplanted and were incubated at 28°C. Root rot severity was evaluated by enumerating the diseased plants after 10 days. Data from the plant experiment were statistically analysed using Mann Whitney test at 5% probability.

**Results**

*Survey on concurrent production of biosurfactants and phenazines in fluorescent pseudomonads*

A collection of 25 phenazine-producing *Pseudomonas* WT strains was tested for their ability to synthesize biosurfactants using the drop collapse technique. Forty % of the phenazine-producing bacteria has the ability to synthesize biosurfactants. Strikingly, all PCN-producing
WT strains are capable of producing biosurfactants except for strain *P. aureofaciens* LMG5004. Presumably, the biosurfactant genes may not be expressed or present in this strain. From the biosurfactant perspective, however, it appeared that all biosurfactant producers synthesize PCN. WT strains that solely produced PCA or 1-hydroxy-phenazine, did not produce biosurfactants.

*Evaluation of in vivo synergy between phenazines and biosurfactants in fluorescent pseudomonads*

The synergistic interaction between phenazines and biosurfactants in the biocontrol of *P. myriotylum* on cocoyam was examined for three different *Pseudomonas* strains: *Pseudomonas chlororaphis* PCL1391, *Pseudomonas* CMR5c, and *Pseudomonas* CMR12a. They were selected based on the fact that they produce both phenazines and biosurfactants. Phenazine mutants of PCL1391, and biosurfactant mutants of CMR5c and CMR12a were tested for the loss of biological control activity. Table 1 shows the percentage of diseased cocoyam plants for each treatment after 10 days.

*Pseudomonas* strains that produce both phenazines and biosurfactants significantly reduced cocoyam root rot. The WT strains PCL1391, CMR5c, and CMR12a significantly suppressed cocoyam root rot. PCL1119, a phenazine mutant of PCL1391 could no longer control cocoyam root rot, although the mutant still produced biosurfactants. The biosurfactant mutants CMR12a 7.97 and CMR5c 9.46 did not longer protect cocoyam plantlets from root rot despite the fact that the strains still synthesized phenazines.

Table 1. Biological control activity of phenazine and/or biosurfactant mutants of three *Pseudomonas* strains against cocoyam root rot

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PCA</th>
<th>PCN</th>
<th>Bio-surfactants</th>
<th>Diseased plants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Control + Pythium</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td><em>P. chlororaphis</em> PCL1391 + Pythium</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>57</td>
</tr>
<tr>
<td><em>P. chlororaphis</em> PCL1119 + Pythium</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>83</td>
</tr>
<tr>
<td><em>Pseudomonas</em> CMR5c + Pythium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>55</td>
</tr>
<tr>
<td><em>Pseudomonas</em> CMR5c 9.46 + Pythium</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudomonas</em> CMR12a + Pythium</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>41</td>
</tr>
<tr>
<td><em>Pseudomonas</em> CMR12a 7.97 + Pythium</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>92</td>
</tr>
</tbody>
</table>

*Discussion*

Phenazines and rhamnolipid-biosurfactants produced by *P. aeruginosa* PNA1 act in synergy in the biocontrol of *Pythium* induced soil-borne diseases. This present study provides evidence that phenazines and biosurfactants produced by fluorescent pseudomonads other than *P. aeruginosa* PNA1 also interact in the biological control of *Pythium* root rot on cocoyam. Furthermore, this work demonstrated that not all phenazine-producing pseudomonads invest in the biosynthesis of biosurfactants. Nevertheless, there appears to be a link between PCN production by a strain and the ability of that strain to synthesize biosurfactants. No such association however, could be found for biosurfactant production in combination with other phenazine compounds. Hence, a causality dilemma arises whether PCN is required for biosurfactant production or the reverse.
PCN is required for biosurfactant production if we assume that PCN is a signalling molecule inducing the expression of biosurfactant genes. Recent studies postulated that phenazine compounds could act as intracellular signalling molecules inducing the expression of certain genes (Dietrich et al., 2006; Maddula et al., 2006). Our finding that PCA producers do not synthesize biosurfactant supports this hypothesis. It remains to be investigated whether PCA producers do not have the biosurfactant genes or if the biosurfactant genes are simply not expressed in these strains.

Biosurfactant is required for PCN production, if we assume that biosurfactant production is crucial for the biological activity of PCN. The octanol-water partition coefficient ($K_{ow}$), which is a measure for the hydrophobicity of a compound, is consistently higher for PCN than for PCA (Price-Whelan et al., 2006). This implies that PCN is more hydrophobic than PCA. In this context, it is plausible that bacteria synthesize biosurfactants to increase the solubility of PCN and consequently its biological activity. The idea that an organism produces biosurfactant to increase the solubility of its own hydrophobic metabolites is not illogical.

Plant experiments on cocoyam demonstrated that mutant strains deficient in either phenazine or biosurfactant production could not provide sufficient biocontrol activity against the root rot pathogen *P. myriotylum*. Only the WT strains significantly suppressed cocoyam root rot. These results indicate that also in these fluorescent pseudomonads, phenazines and biosurfactants act in synergy.

**Acknowledgements**

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**References**


PGPR-induced systemic resistance in rice

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Abstract: Induced resistance can be defined as the phenomenon by which plants exhibit increased levels of resistance to a broad spectrum of pathogens by prior activation of genetically programmed defense pathways. To date, molecular biology research aimed towards understanding induced resistance mechanisms has focused mainly on dicotyledoneous model plant species such as Arabidopsis thaliana and tobacco. Conversely, in the class of Monocotyledoneae, including the most important agronomic cereals, molecular information on chemically and biologically induced resistance mechanisms is largely missing. The aim of our research is to assess whether selected non-pathogenic plant-growth promoting rhizobacteria (PGPR), which have been shown previously to mount induced systemic resistance (ISR) in dicots, are also capable of triggering ISR in rice against major fungal pathogens such as Magnaporthe grisea and Rhizoctonia solani and if so, to elucidate the bacterial determinants and plant defense pathways involved in this process.

Although a clear protective effect against M. grisea was observed, Pseudomonas aeruginosa 7NSK2 proved unable to consistently reduce R. solani infection severity. Whereas 7NSK2 activates ISR in dicots through a synergistic interaction of the siderophore pyochelin and the antibiotic pyocyanin, only mutations interfering with pyocyanin production led to a significant decrease in ISR to M. grisea in rice. Moreover, pyocyanin concentrations as low as 50 pM applied to the roots of hydroponically grown rice seedlings mimicked the ISR effect. Intriguingly, pyocyanin-deficient mutants, unlike the wild-type strain, significantly reduced sheath blight disease severity while pyocyanin feeding favoured subsequent infection by R. solani. Biochemical analyses in a gnotobiotic rice-growing system demonstrated that application of redox-active pyocyanin to the roots triggers reiterative H$_2$O$_2$ microbursts in planta. Although root treatment with pyocyanin, at least in the pico- and nanomolar range, did not induce visible cell death by itself, nor in local nor in systemic tissue, a marked increase in the number of HR-expressing epidermal penetration sites was observed in response to infection with M. grisea. Furthermore, pyocyanin feeding provoked intense burning of epidermal cells ahead of R. solani invasion. Addition of the antioxidant ascorbate to the pyocyanin feeding solution abrogated these pyocyanin-mediated host responses. Hence, transient pyocyanin-induced H$_2$O$_2$ microbursts and related HR-like cell death act as a double-edged sword in 7NSK2-mediated ISR. In line with this concept, cytological studies revealed that Serratia plymuthica strain IC1270, which is a potent inducer of ISR to M. grisea but fails to mount resistance to R. solani, primes rice seedlings for potentiated systemic generation of reactive oxygen species in response to wounding or pathogen infection.

Interestingly, root treatment with Pseudomonas fluorescens strain WCS374 effectively protected rice plants against both M. grisea and R. solani. A combination of mutant analysis and experiments using purified compounds demonstrated that the bacterial determinants of WCS374-triggered ISR are the siderophores pseudobactin and salicylic acid. Currently, we are investigating the molecular mechanisms underlying WCS374-mediated ISR.

This study provides new insights into the molecular biology underlying the phenomenon of ISR in the model plant rice and sheds new light on the differential beneficial effect of the oxidative burst as defense response against hemibiotrophic (M. grisea) and necrotrophic rice (R. solani) pathogens.
PGPR-induced systemic resistance: activity of amphiphilic elicitors and structural analogues on different plant species

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Abstract: Some non-pathogenic microorganisms can induce disease resistance mechanisms expressed systemically in the host plant thereby rendering it less susceptible to further attack by pathogens. In this study, we have investigated the role of bacterial compounds as elicitors of the induced systemic resistance. Lipopeptides, especially surfactin and fengycin, produced by Bacillus subtilis strains are able to stimulate bean and tomato plants and decrease the impact of subsequent pathogen infection. Preliminary experiments on tobacco cells showed that surfactine induces some modifications in the phenylpropanoid pathway. Amphiphilic properties of lipopeptides and NABD, the elicitor isolated from Pseudomonas putida strain BTP1, could be responsible for their activities on plant cells.

Key words: lipopeptides, rhizobacteria

Introduction

Utilization of some beneficial rhizobacteria to stimulate plant growth or to protect them against pathogen attack represents an increasing interest. These rhizobacteria can inhibit pathogen development via two major mechanisms. The first is direct antagonism through antibiotic production, site exclusion or siderophore-mediated competition for iron uptake (Zahir et al., 2004). The second is through the stimulation of plant defenses rendering the host less susceptible to a subsequent pathogen attack. By analogy to the well known pathogen-derived SAR phenomenon (Durrant & Dong, 2004), the “immunization” of plant by non-pathogenic microorganisms was termed induced systemic resistance (ISR) (van Loon et al., 1998). Some molecules such as cell surface components (Meziane et al., 2005), antibiotics (pyocyanin/pyochelin, 2,4-diacetylphloroglucinol) (Audenaert et al., 2002) or iron-regulated metabolites (salicylic acid, pyochelin or pyoverdines) (De Meyer et al., 1999), are now know to play a major role in ISR activation. However, even if the phenotypic effects are similar in SAR and ISR, the molecular events associated with the ISR and especially the recognition mechanisms of bacterial elicitors are less well known.

In this context, we have studied for several years a Pseudomonas putida strain (BTP1) for its ability to trigger ISR in cucumber, bean and tomato (Ongena et al., 2002). Previous investigations have demonstrated that ISR activity of BTP1 on bean was due to a new molecule, a N-alkylated benzylamine derivative (NABD) (Ongena et al., 2005). This compound contains a benzylamine core substituted by three alkyl groups. Here we summarize the results obtained by testing NABD and lipopeptides on different plants for their ISR eliciting effect. With the aim to identify its mode of action and its plant specificity, we have investigated the availability of pure NABD (synthesised by organic chemistry) to induce a biological response on cucumber and tomato. In parallel, the action of lipopeptides, produced
by some *Bacillus subtilis* strain and already known to have some direct antagonisms on pathogen (Ongena et al., 2005), was investigated on plants for their possible ISR stimulation. Finally, one of both lipopeptide, surfactine, was also tested on tobacco cell in vitro culture.

**ISR activity of Bacillus lipopeptides**

ISR experiments were realized by using *Bacillus subtilis* 168 (wild type), a very poor producer of lipopeptides, and three derivatives constructed to over-produce each lipopeptide type.

![Figure 1. Infection rate caused by *B. cinerea* on bean (A) and tomato (B) plants. Plants were treated at the root level with living cells of *B. subtilis* strains and infected on leaves with a conidia suspension of *B. cinerea*.

"a" marked Bars are statistically similar to the controls.

As observed with bean and tomato, *B. subtilis* 168 (BCWT), is not able to induce a significant reduction of the disease. However, application of lipopeptide overproducing mutants BC21 and BC25 that both produce surfactin in large amounts (respectively 750 and 697 mg/L) yielded significantly decreased disease symptoms. Disease reduction was also observed but to a lower extent with BC27 that exclusively overproduces fengycin (452 mg/L). The tests of pure lipopeptides on bean also revealed a higher ISR inducing activity of surfactin compared to fengycin (data not shown). On both plants, treatment with BC25 that overproduces both surfactin (697 mg/L) and fengycin (434 mg/L) yielded a higher albeit not significantly different protective effect suggesting a synergistic effect of the two molecules.

**Effect of surfactin on tobacco cells**

The activity of surfactin on plant cells was further tested in tobacco cell suspension cultures. Sampling was realized at different time and ethyl acetate supernatant extracts were analysed by HPLC-ESI-MS. Preliminary analyses revealed an accumulation of coumaric, cinnamic, OH-benzoic and salicylic acids associated with a decrease in phenylalanine, coumaric and ferulic acids. Moreover, other unidentified compounds specifically appear in the culture supernatant of surfactin-treated cells. These preliminary results strongly suggest significant changes in the phenylpropanoid pathway particularly involved in plant defence response.

**Resistance induced in plants by NABD from *Pseudomonas putida* BTP1**

The ISR activities of pure NABD, semi-purified supernatant extract and living cells of *Pseudomonas putida* BTP1 were compared on three different plants. As shown in figure 2, the
semi-purified supernatant extract induces a disease reduction at the same level as the one provided by living cell on bean (A), cucumber (B) and tomato (C).

Figure 2. Infection rate on bean (A) cucumber (B) and tomato (C) plants treated at the root level with living bacteria (cells), semi-purified supernatant extract of *Pseudomonas putida* BTP1 culture (Extract), synthetic NABD (Pure NABD) and benzylamine (Benzylamine). Bean and tomato were infected with a spore suspension of *Botrytis cinerea*. Cucumber cotyledons were infected with a spore suspension of *Colletotrichum lagenarium*. Bars marked with “a” are not statistically different from the controls.

Likewise, root treatment with pure NABD also statistically decreases the disease impact on bean and cucumber leaves. However, the lower activity of NABD observed on tomato suggest the presence of other(s) compound(s) with ISR inducing effect in the supernatant extract. Interestingly, treatment with benzylamine, representing the core of NABD, induces a similar protection level compared to cells, extract and pure NABD on bean and cucumber suggesting a crucial role of this group for the biological activity of the whole molecule.

To our knowledge, the ISR elicitor synthesized by BTP1 has not yet been described among *Pseudomonas* products. The NABD molecule is not structurally related to the other ISR determinants and its discovery is in support of a wider variety of bacterial determinants.
for ISR than is reflected by the limited number of such molecules reported to date. The *Bacillus* lipopeptides surfactin and fengycin also constitute a novel class of compounds from non-pathogenic bacteria that can be perceived by plant cells as signals to initiate defense mechanisms. These structures may represent epitopes recognized by specific receptors in the membrane of root cells. However, striking similarities are shared by NABD and lipopeptides: an amphiphilic nature dictated by the presence of a long hydrophobic alkyl chain and a charged moiety (surfactant properties). Given the ability of such molecules to insert into lipid layers, they could also act by creating some disturbance or channeling in the plasma membrane of plant cells that may in turn activate a cascade of molecular events leading to the expression of defensive responses.

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Potential and use of molecular techniques to understand the mechanisms of action of fungal biocontrol agents

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Abstract: Biological control of fungal plant pathogens appears to be an attractive and realistic approach and, so far, numerous micro-organisms have been identified as biocontrol agents. Up to now, numerous efforts have been made to understand the mechanisms of action of fungal biocontrol agents. Microbiological, microscopic and biochemical techniques have been used for many years. They improved the knowledge on the mechanisms of action but failed to fully demonstrate them. More recently, the development of molecular techniques has provided to the researchers innovative and alternative tools to understand and to demonstrate the mechanisms involved in the biocontrol properties. So far, more than 60 publications used molecular techniques to study the mechanisms of action of biocontrol agents through targeted or non targeted gene isolation, gene expression study, gene inactivation, gene over-expression and regulation factors study. These techniques allowed significant advances in the understanding of the mechanisms of action involved in biocontrol properties. Furthermore, they also fully demonstrated the involvement of targeted mechanisms of actions of some biocontrol agents. The objectives of this presentation are to review the techniques already used and to evaluate their potential and their limitations for studying the mechanisms of action. Finally, this review aims to provide a guide for the researchers who want to study the molecular basis of the biocontrol properties of their biocontrol agents.
Evaluation and mode of action of *Trichoderma* isolates as biocontrol agents against plant-parasitic nematodes

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Abstract: Seven *Trichoderma* isolates [T. asperellum (T-34, T-203, T-44 & T-GH11), T. atroviride (T-IMI 206040), T. hamatum (T-382) and T. harzianum (T-248)] exhibited the ability to biocontrol plant-parasitic nematodes such as the root-knot nematodes, *Meloidogyne incognita* and *M. javanica* (endo-parasites). This nematicidal activity was examined in vitro, in growth-chambers and in micro-plots, with 3 crops (cucumber, lettuce and tomato) and in two different soil types. All 7 isolates increased top fresh weights and reduced galling indices, as compared to nematode-infected non-treated plants. The fungus-nematode interactions included several direct and indirect mechanisms that took place in soil and in plant roots. Parasitism of *Trichoderma* on different nematode life-stages was examined in vitro, and in planta on fungus-treated tomato plants using a constitutively expressing GFP (green fluorescent protein) construct of T-203. Both pre-infective, infective second-stage juveniles (J2) and eggs of *Meloidogyne* were colonized by the fungus. It also colonized J2 penetration holes within the roots. Egg-masses, covered by the gelatinous matrix (GM), and females dissected from *Trichoderma*-treated roots were found to be colonized by the fungus. GM-coated nylon fibers were used to demonstrate the important role of the GM in fungal spore attachment and induction of fungal parasitic growth patterns such as coiling. Fucose-specific antibody and a fucose-binding lectin enhanced spore attachment to the nematode surface. Proteinase Prb1 in *T. atroviride* and other proteases in different *Trichoderma* isolates were involved in nematode parasitism. The induction of chitinolytic activities during parasitism on nematode eggs and egg-masses was demonstrated by using GFP-reporter fungal constructs of the endochitinases CHIT36 and CHIT42 and the N-acetyl-glucosaminidases CHIT102 and Nag1, in *T. asperellum*-203 and *T. atroviride*. The role of induced systemic resistance in the biocontrol activity was evaluated in a split-root system: significant reductions in root penetration, nematode development inhibition within all life stages, galling indices and subsequent retardation in reproducibility, were recorded in the root halves that were not directly treated with *Trichoderma*.

Key words: chitinolytic and proteolytic enzymes, gelatinous matrix, lectins, *Meloidogyne incognita*, *M. javanica*, *Trichoderma asperellum*, *T. hamatum*

Introduction

Plant-parasitic nematodes cause great economic losses to agricultural crops worldwide. Root-knot nematodes (RKN, *Meloidogyne* spp.) such as *M. incognita* and *M. javanica*, are among the major limiting factors in the production of field and plantation crops. These species are sedentary root endoparasites and polyphagous. There are very few biocontrol products available for use against nematodes; therefore, there is a need to develop efficient biocontrol agents and to understand their mode of action.
The RKN second-stage juveniles (J2s) penetrate the host roots and develop within them. They induce a cascade of changes in the host plant, which lead to the formation of giant cells and galls. About one month after J2s penetration, the females lay out egg masses that contain gelatinous matrix (GM). The GM is usually considered as an egg-mass protecting material from the external environment (Sharon et al., 1993). Several reports on the interactions of Trichoderma species and plant-parasitic nematodes have been published (Saifullah & Thomas, 1996; Windham et al., 1989).

Following preliminary studies in our laboratories (Sharon et al., 2001; Spiegel and Chet 1998), several Trichoderma species and isolates were evaluated as biocontrol agents against two RKN species under growth-chamber conditions. Fungal treatments exhibited significant biocontrol activity. Aiming to improve the biocontrol process, modes of action of the fungus against the nematodes have been investigated.

Material and methods

Nematodes
The root-knot nematode species, Meloidogyne incognita and M. javanica were propagated on tomato plants (Lycopersicon esculentum Mill cv. Growdena, and var. 144, in Belgium and Israel, respectively), grown in the growth chamber. Monoxenic cultures of M. javanica were cultured aseptically in Petri dishes on excised tomato roots. Those cultures were used to obtain nematode egg-masses and gelatinous matrix (GM). Separated GM-free eggs were extracted from infected roots with sodium hypochlorite (NaOCl 0.5%). Pre-infective second-stage juveniles (J2) were hatched from those eggs or directly from egg-masses.

Trichoderma isolates and preparations
Trichoderma asperellum-203, T. asperellum-44, T. asperellum- GH11, designated herein as T2, T3 and T5, have been originally grown, identified, tested and assimilated to STRI in Belgium from ARO, Israel. T. atroviride (T-IMI 206040), T. harzianum (T-248) and transformed T. atroviride lines which were provided by Prof. A. Herrera-Estrella from Mexico, were used in Israel. T. asperellum-34 and T. hamatum-382, designated herein as T34 and T382, have been originally grown in STRI, Belgium. Fungal cultures were grown on potato dextrose agar (PDA) (Difco) and used in the experiments as solid preparations grown on wheat Dark Sphagnum peat (DSP)-bran according to the method described by Sivan et al. (1984). The preparations contained ca. 10⁸ CFU/g.

Evaluation of nematicidal activity of different Trichoderma isolates in two different naturally-infested soil: growth chamber and micro-plot experiments
Two-successive ‘life-cycle’ experiments were performed in the growth chamber. In the first cycle, Trichoderma preparations (1%, w/w) were mixed in 0.5, 3 or 4 L pots with two different sandy soils in Belgium and in Israel [pH (KCl) – 5.1 and 7.0; EC (µS/cm) – 61 and 93; Total % of dry C and N – 1.64, 0.1, and 0.07, 0.01]. Soil was naturally-infested with second-stage juveniles (J2) and eggs of M. incognita or M. javanica and the pots were located in a temperature-controlled growth chambers (27 °C±2; 70% humidity and 12 h/12 h light/dark). Ten days after Trichoderma assignment to soil, cucumber, lettuce or tomato plants were planted. Non-treated nematode-infested soils served as a control. Plants were fertilized weekly with a nutrient solution, and watered as needed with plain tap water. In most experiments, 35 days after seedlings’ exposure to nematodes, plants were removed, and top fresh weights (TFW) and root-galling indices (GI) were evaluated on a 0 - 5 scale (Bridge & Page, 1980). For the second-cycle experiment, soil used in the first-cycle was relocated in the pots and plants’ seedlings were planted immediately without further treatments. Thirty days later, plants were harvested and TFW and GI were evaluated as described before. The
experiments were conducted at least twice with seven replicates per treatment. Results were analyzed statistically with GraphPad program (Copyright 1992-1998 GraphPad Software Inc.), using one-way ordinary ANOVA tests followed by Tukey-Kramer Multiple Comparisons post tests.

**Parasitism bioassays**

Bioassays of attachment and parasitism on various life stages of *M. javanica* by *Trichoderma* were performed, *in vitro*, in 96-well plates containing minimal medium-based diluted potato dextrose broth (PDB). Monoclonal antibody (MAb) MISC was provided by Prof. M.A. McClure, USA.

**Biomimetic system on nylon fibers**

This system was based on the method developed by Inbar & Chet (1992). Nylon fibers were treated with GM suspension and controls were treated with bovine serum albumin (BSA).

**Enzymatic activity**

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12%, pre-cast, casein-containing gels (BioRad Laboratories).

**Split-root experiments**

Experiments were conducted in 1.5-liter pots divided into halves by a central vertical barrier. The root systems of tomato or cucumber speed-seedlings were split between two halves of the pot. The soil on one side of the pot was treated with *T. asperellum*-203 at a concentration of 1% (w/w) at the time of planting. One week later, the soil was inoculated with 2000 J2s per half-pot, in one or both halves.

**Results and discussion**

*Biocontrol evaluation in growth-chambers and micro-plots*

During most of the growth-chamber and micro-plot experiments, when nematode-infection rate (GI) was moderate or low (1-3), all *Trichoderma* species and isolates exhibited biocontrol activity against *Meloidogyne*. In these occasions, fungal treatments significantly increased top fresh weights and reduced galling indices and egg numbers as well as hatching rates, as compared to nematode-infected non-treated plants. In high inoculation rates (GI 4 - 5), two isolates of *Trichoderma* revealed outstanding biocontrol activity as compared to other fungal isolates: isolates T3 & T5 in lettuce and tomato experiments, in both first and second cycles (Fig. 1), and isolates T3 & T382 in the cucumber experiments. T2 isolate which has been very efficient in its activity against *Meloidogyne* in the growth-chamber and micro-plot experiments in Israel (Fig. 2), failed to do so in the tests run in Belgium; it may be attributed to the different soil types.

**Modes of action**

Egg-masses and females of *M. javanica*, dissected from T-203-treated tomato roots from soil experiments, were found to be colonized by the fungus. In *in vitro* tests, the fungus parasitized egg-masses, their originated eggs and J2 after fungal spores were attached to the various life stages. A biomimetic system, based on GM-coated nylon fibers, demonstrated the important role of the GM in fungal spores’ attachment and induction of fungal parasitic growth patterns such as coiling. Fucose-specific monoclonal antibody, which binds to *M. javanica* J2 and egg surfaces, enhanced spores’ attachment to the nematode life stages and improved the fungal parasitism when incorporated into the *in vitro* parasitism bioassay system. This enhancement was facilitated by the bilateral binding of the antibodies to the nematodes and to the fungal spores. The fucose-specific lectin *Ulex europaeus* agglutinin-I (UEA-I) specifically enhanced spore attachment to nematodes and caused spore agglutination; this behavior resembled that of the antibody.
Proteinase-transformed lines that contain multiple copies of prb1 gene, and an inducible-GFP reporter construct, were used. Line P-2 exhibited improved biocontrol capacity in soil and against all nematode life-stages tested in vitro. This proteinase was induced during fungal parasitism on the various life-stages of M. javanica. Examination of the activity of this proteinase, after electrophoretic separation on zymogram gels, revealed enhanced proteolytic activity during T. atroviride parasitism on the nematodes. The induction of chitinolytic activities during parasitism on nematode eggs and egg-masses was demonstrated by using GFP-reporter fungal constructs of the endochitinases CHIT36 and CHIT42 and the N-acetyl-glucosaminidases CHIT102 and Nag1, in T. asperellum-203 and T. atroviride. These enzymes are probably involved in degradation of the chitin layer in the eggshells.

In a split-root system, significant reductions in J2 penetration to roots, nematode development within all life stages, galling indices and subsequent retardation in reproducibility, were recorded in the root halves that were not directly treated with Trichoderma. These results indicate the potential involvement of systemic induced resistance mechanisms in the nematode biocontrol process.
References


The increase in endochitinases and β-1,3-glucanases in the mutant Th₆₅₀-NG7 of the *Trichoderma harzianum* Th₆₅₀, improves the biocontrol activity on *Rhizoctonia solani* infecting tomato

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**Abstract:** The mutant Th₆₅₀-NG7 obtained after treatment of a wild isolate of *T. harzianum* (Th₆₅₀) with N-methyl-N-nitro-N-nitrosoguanidine (NG), increased two and four fold total β-1,3-glucanase and endochitinase activities, respectively, and improved the biocontrol activity on *R. solani*. Glasshouse assays showed that Th₆₅₀-NG7 prevented the 60 and 20% mortality observed in tomato plants of cvs. 92.95 and Gondola respectively, as a consequence of their inoculation with high pressure of *R. solani* and the parental strain (Th₆₅₀). Results show a correlation between biocontrol ability of Th₆₅₀-NG7 and secretion of *R. solani* cell wall hydrolyzing enzymes.

**Key words:** crown rot, *Lycopersicon esculentum*, root rot

**Introduction**

The need to find alternatives for the control of *R. solani* that causes crown and root rot and death in tomato plants as well as for other phytopathogens that also infect this crop, focused our work in the finding and improvement of local isolates of *T. harzianum*, a well known biocontrol agent (Papavizas, 1985; Rey et al., 2000). Th₆₅₀, a selected local wild strain of *T. harzianum* that controlled *R. solani* development at the laboratory level (Arias, 2005), was previously characterized related to its biocontrol mechanisms (Montealegre et al., 2005). On the other hand, the use of NG had proved useful to obtain mutants from *T. aureoviride*, which improved the secretion of enzymes involved in biocontrol: endochitinases and endoglucanases (Zaldívar et al., 2001). Therefore, we decided to use a similar approach on Th₆₅₀, to obtain new strains that could behave better as biocontrol agents than the parental one.

**Material and methods**

**Tomato cultivation conditions**

Tomato seedlings from the cvs. 92.95 and Gondola were planted in 2.3 L pots containing sterile soil and inoculated later with 21 g of a *R. solani* (strain 618) per pot, equivalent to 2.15×10⁵ colony forming units per gram of soil (CFU/g soil) (Santander, 2001) and with Th₆₅₀ and Th₆₅₀-NG7 selected on the basis of fungal growth and secreted enzyme activities (Pérez & Morales, 2003). The *T. harzianum* strains were applied in sodium alginate pellets (Besoain et
al., 2004) in dosages of 1.7 g pellets/L soil. Treatments (Table I) were compared to controls run with *R. solani* 618 (*T*₀) and with *R. solani* 618 + Pencycuron (0.15 mL/pot) (*T*₁). Evaluations included: a) plant mortality (expressed as %), b) canker formation at stem base (expressed as qualification in a scale of one to five) and c) root development (Kruskal-Wallis non-parametric test, by pair comparisons according to a Mann-Whitney test).

**Fungal isolates, culture conditions and formulation in pellets**

*R. solani* strain 618 (AG-4), and *T. harzianum* strain Th₆₅₀, were obtained and cultured as described (Montealegre et al., 2005). Th₆₅₀-NG7 was obtained as in Zaldívar et al. (2001) and was cultured in the same conditions. Pellets were formulated with Th₆₅₀ and Th₆₅₀-NG7 as in Besoaín et al. (2004).

**Analysis of Th₆₅₀-NG7**

Supernatants from Th₆₅₀-NG7, grown in liquid Mandels medium using *R. solani* cell walls (Pérez et al., 2002) were characterized by: a) native PAGE [secreted endochitinases (E-chiti), β-1,3-glucanases (Glcases) and endoproteases (E-Prases) (Pérez et al., 2002)]; b) IEF [secreted E-chiti (Pan et al., 1991)] and c) Spectrophotometric analysis [Glcases and E-Prases] using specific Megazyme® substrates. They were compared with supernatants from Th₆₅₀, the parental strain.

**Results and discussion**

Th₆₅₀-NG7, the new strain obtained after treatment of Th₆₅₀ with NG, decreased canker qualification, increased root growth and prevented mortality caused by *R. solani* in tomato plants from Cv. 92.95 (Table 1). The prevention of mortality was also observed after treatment of Cv. Gondola with this same strain, although canker qualification or root growth did not show significant differences from controls run in the absence or in the presence of fungicide.

**Table 1. Effectiveness of Th₆₅₀-NG7 on *R. solani* infecting tomato plants under glasshouse conditions**

<table>
<thead>
<tr>
<th></th>
<th>A) Cv. 92.95</th>
<th></th>
<th>B) Cv. Gondola</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T</em>₀¹</td>
<td><em>T</em>₁²</td>
<td>Th₆₅₀</td>
</tr>
<tr>
<td>Canker qualification</td>
<td>4.8 d</td>
<td>2.4 b,c,d</td>
<td>4.2 d</td>
</tr>
<tr>
<td>Root growth</td>
<td>0 b</td>
<td>1.4 b</td>
<td>1.2 b</td>
</tr>
<tr>
<td>Mortality</td>
<td>100</td>
<td>20</td>
<td>60</td>
</tr>
</tbody>
</table>

Canker, root growth and mortality of tomato plants of cvs. 92.95 (A) and Góndola (B) tomatoes v/s treatments [1/. *T*₀ (only *R. solani* 618). 2/. *T*₁ (*R. solani* + Pencycuron (0.15 mL/pot)]. Canker qualification (0: =%; 1: <5%; 2: 5-30%; 3: 30-60%; 4: 60-90%; 5: >90%) and root growth qualification (0: No growth; 1: Poor growth; 2: Moderate growth; 3: Good growth and 4: Very good growth) evaluated using the Kruskal-Wallis non-parametric test, by pair comparisons according to a Mann-Whitney test. Means in a file with the same letter indicate no statistical differences, according to Tukey multiple comparison tests at *P*≤0.05.
Treatment with the parental strain \( T_h_{650} \) showed no differences between cvs. 92.95 and Gondola, except for the 40 and 50% decreases in mortality, respectively. These results correlate well with the 150% increase in growth of \( T_h_{650}-NG7 \) (data not shown), and with the E-chiti (4-fold increase) and Glcases (c.a. 2-fold increase) activities secreted, when compared to the parental strain (Table II). Native PAGE run at pH 4.4 of supernatants of \( T_h_{650}-NG7 \) showed the presence of the same two bands of E-chiti already described for \( T_h_{650} \) (Montealegre et al., 2005). In addition, it also showed a single band of E-chiti after PAGE run at pH 8.8. IEF, run in a range of pH 3 to 10, showed different number of bands for \( T_h_{650}-NG7 \) (pI of 5.1, 6.5, 7.2 and 8.0) and \( T_h_{650} \) (pI of 6.5 and 7.2). The band at pH 6.5 splits in four bands, both in \( T_h_{650} \) and in \( T_h_{650}-NG7 \) after IEF in the range of pH 5 to 7 (Table 2).

Table 2. Characterization of \( T_h_{650}-NG7 \) grown in Mandels liquid medium in the presence of \( R. \ solani \) cell walls

<table>
<thead>
<tr>
<th>E-chiti</th>
<th>PAGE: N° of bands</th>
<th>Glcases</th>
<th>E-Prases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity(^1)</td>
<td>pH 4.3</td>
<td>pH 8.8</td>
<td>pH 3-10</td>
</tr>
<tr>
<td>( T_h_{650} )</td>
<td>1(^a)</td>
<td>2(^3)</td>
<td>1</td>
</tr>
<tr>
<td>( T_h_{650}-NG7 )</td>
<td>4(^b)</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Enzyme activity expressed as: \(^1\)Integrated Optical Density obtained using the Gel Proanalyzer Program 3.1. IOD of one was established in the program for E-chiti in \( T_h_{650} \); \(^2\)(abs590nm min\(^{-1}\)mg protein\(^{-1}\)) x 10\(^3\); \(^3\)Taken from Montealegre et al., 2005. Means in a column with the same letter indicate no statistical differences, according to Student’s test at \( P \leq 0.05 \).

Glcases activity also increased two fold in \( T_h_{650}-NG7 \), but E-Prases remained the same thus suggesting a null role in biocontrol activity of this strain against \( R. \ solani \). Results obtained after treatment of tomato plants with \( T_h_{650}-NG7 \) correlate well with the increase both in E-chiti and in Glcases in this strain. A correlation between activity and number of E-chiti isoenzymes was described for a wild strain of \( T. \ harzianum \) (Th11) and its biocontrol effect on \( P. \ lycopersici \) (Pérez et al., 2002). The increase of E-chiti and of Glcases in \( T_h_{650}-NG7 \) could account for the improvement of the biocontrol activity, where these enzyme activities are cooperating in the degradation of the \( R. \ solani \) cell wall.

**Acknowledgements**

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Pathogenicity genes in the sclerotial mycoparasite *Coniothyrium minitans*

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Abstract: *Coniothyrium minitans* is a fungal biocontrol agent of the plant pathogen *Sclerotinia sclerotiorum*. It attacks the sclerotia of the pathogen in the soil, reducing the inoculum potential and decreasing disease. Although its ability to attack sclerotia has been known for a long time, there is little information concerning the process of sclerotial colonisation at the molecular level. We are using a range of gene identification and characterisation approaches to dissect the mechanisms of sclerotial mycoparasitism in this interaction. The first approach used suppression subtraction hybridisation between cDNA from *C. minitans* grown in culture and *C. minitans* colonising sclerotia of *S. sclerotiorum*. A subtracted library of 672 clones containing cDNA fragments of putative upregulated genes was established. Sequencing of these cDNA clones and bioinformatics analysis led to the identification of 251 ESTs and assignment of putative functions. Dot blot and virtual Northern analysis showed different levels of upregulation of various *C. minitans* genes during sclerotial colonisation. The second approach involved insertional mutagenesis of *C. minitans* using REMI and T-DNA tagging. Nine pathogenicity mutants were obtained from a panel of over 4000 transformants. Genes with similarity to the *PIF1* helicase of *Neurospora crassa* and *PTH11* of *Magnaporthe grisea* were amongst those identified. Molecular characterisation and analysis of these pathogenicity mutants is now underway. The final approach has been to isolate genes putatively involved in signalling and colonisation from sequence information available in other pathogenic systems. Using PCR based methods and a genomic macroarray, *pkaC*, *pmk1*, and *cmg1* genes have been obtained from *C. minitans*. Characterisation of some potentially key genes has now begun and gene silencing and complementation studies to investigate their role in sclerotial parasitism have been initiated.

Key words: biological control, *Sclerotinia sclerotiorum*, SSH

Introduction

Environmental concerns about the use of chemicals in crop protection have reduced the number of active ingredients on the market, including loss of the key soil sterilant methyl bromide, and problems associated with control of soilborne plant pathogens have increased. Not surprisingly, sclerotium forming pathogens are becoming especially problematic because their sclerotia (resting bodies) can survive for many years in the soil in the absence of a host and can be very difficult to destroy. Thus fungi that act as sclerotial mycoparasites are of particular interest for use as biological disease control agents. Several commercial products are on the market for control of soilborne plant pathogens (Whipps & Lumsden, 2001) including some *Trichoderma* species and *Coniothyrium minitans* that have activity against sclerotium forming fungi such as *Sclerotinia sclerotiorum* which has a host range of over 400 species (Boland & Hall, 1994). However, even though their practical use against *S. sclerotiorum* has been demonstrated repeatedly (Budge et al., 1995; Gerlagh et al., 1999; Jones et al., 2004) remarkably little is known about the genes involved in sclerotial
pathogenesis itself, despite the increasing literature associated with mycoparasitism involving hyphal hyphal interactions (e.g. Staeyert et al., 2003; Mukherjee et al., 2003; Carpenter et al., 2005). Consequently, we have been examining the role of genes expressed by C. mimitans associated with infection of sclerotia of S. sclerotiorum.

Material and methods

Suppression subtraction hybridisation (SSH)
Subtractive hybridisation was performed between the driver (10 day old Potato Dextrose Agar (PDA) grown C. mimitans Conio) and the tester (10 day old sclerotial colonizing C. mimitans) using the Clonetech PCR select cDNA subtraction kit. The enriched cDNA populations from SSH were cloned using the TOPO TA cloning kit and TOP10 Escherichia coli cells (Invitrogen Ltd, UK) were transformed with the cloned DNA library and recombinant colonies selected based on the blue/white screening. A subtracted library consisting of 672 clones was established and was sequenced using Templiphi amplification (Amersham Biosciences) kit. Following assembly and editing of DNA sequence contigs using DNAStar (Lasergene software, DNAStar Inc.) and Vector NTI (Invitrogen) packages, unisequences were identified.

Insertional mutagenesis using restriction enzyme mediated integration (REMI) and Agrobacterium tumefaciens mediated transformation (ATMT)
Coniothyrium mimitans strain Conio was transformed to hygromycin-resistance by random integration of transforming DNA, using either plasmid pAN7-1 (REMI) or T-DNA derived from plasmid pBIN7.1 (ATMT) (Rogers et al., 2004). For REMI transformation, protoplasts of C. mimitans were produced from 3-day old broth grown (static) mycelia using a mixture of cell-wall degrading enzymes (glucanase, cellulase and chitinase). Plasmid DNA was added to the purified protoplasts along with the restriction enzyme HindIII. For ATMT, C. mimitans conidia were incubated on PDA for 2-days, before co-cultivation on induction media with the appropriate Agrobacterium strain for a further two days prior to selection. Transformation efficiency for both systems, while not directly comparable, yielded similar numbers of transformants: REMI yielded c. 32 transformants/µg DNA, a two-fold increase over non-REMI transformation, while ATMT gave a maximum of 37.5 transformants/5x10⁵ germlings.

A panel of 3000 REMI and 1000 ATMT transformants were screened for loss of pathogenicity to sclerotia of S. sclerotiorum using a rapid microtitre plate based assay. Six of these were non-pathogenic, with results identical to the negative control (un-inoculated), with the remaining three displaying reduced sclerotial pathogenicity compared to wild-type infection. Sequences flanking the single site of insertion in four of these tandem-copy mutants were recovered using plasmid rescue. Genomic DNA was digested with a number of enzymes that were selected to cut once within the plasmid, and therefore would result in the recovery of one flank from the insertion site. Following ligation, transformation into E. coli and selection for ampicillin resistance, clones were recovered containing the fragment of interest. Fragments were between 1 kb and 2.7 kb in length and all four contained predicted ORFs, suggesting that in these mutants the plasmid copies had integrated either within or close to potential pathogenicity genes. Sequence data was used to construct short 400-500bp probes that were radioactively labelled and used to screen a macroarray of the C. mimitans genomic cosmid library. Cosmids were identified that hybridised to each of the four probes and were used to recover the wild-type sequence of the regions disrupted in the pathogenicity mutants.

Heterologous probing to isolate C. mimitans pathogenicity genes
A series of potential pathogenicity genes including selected cell wall degrading enzymes and those involved in signalling were identified from the literature and specific PCR primers
designed for these genes based on sequences available in the databases. Probes were subsequently prepared and used to interrogate *C. minitans* genomic library macroarrays and the putative heterologous genes cloned and sequenced from the appropriate cosmid clones.

**Results and discussion**

**Suppression subtraction hybridisation (SSH)**
Following searches of the sequences obtained from SSH using various databases such as COGEME, SGD and MIPS using tblastX or blastX, 251 unisequences were identified and putative functions assigned (Table 1).

<table>
<thead>
<tr>
<th>Putative function</th>
<th>% of sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td>25</td>
</tr>
<tr>
<td>Energy</td>
<td>5</td>
</tr>
<tr>
<td>Transcription</td>
<td>3</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>6</td>
</tr>
<tr>
<td>Protein destination</td>
<td>4</td>
</tr>
<tr>
<td>Transport facilitation</td>
<td>7</td>
</tr>
<tr>
<td>Cell communication and signal transduction</td>
<td>2</td>
</tr>
<tr>
<td>Cell rescue, defence, death and aging</td>
<td>25</td>
</tr>
<tr>
<td>Cellular organization</td>
<td>1</td>
</tr>
<tr>
<td>Retro elements</td>
<td>1</td>
</tr>
<tr>
<td>Transmembrane proteins</td>
<td>1</td>
</tr>
<tr>
<td>Unknown/ hypothetical</td>
<td>12</td>
</tr>
<tr>
<td>Poor or no hit</td>
<td>12</td>
</tr>
</tbody>
</table>

Insertional mutagenesis using restriction enzyme mediated integration (REMI) and Agrobacterium tumefaciens mediated transformation (ATMT)
A total of nine pathogenicity mutants were identified from the panel of 4000 transformants, a frequency of 0.225%. The four tandem copy mutants examined all contained putative pathogenicity genes since ORFs were present at the sites of integration. Full length sequences were obtained for two of these mutants from a cosmid library. BLAST searches identified genes with similarity to: a PIF1 helicase gene of *Neurospora crassa*, with roles in maintenance and repair of DNA along with a pisatin demethylase gene from *Nectria haematococca*, involved in the inactivation of a plant phytoalexin; and a PTH11 gene of *Magnaporthe grisea* involved in signalling; and a gene of unknown function.

**Heterologous probing to isolate *C. minitans* pathogenicity genes**
Heterologous probing of the *C. minitans* macroarray enabled putative signaling genes *Cm pmk1* and *Cm pkaC* to be obtained, along with cell wall degrading enzymes such as chitinase, glucanase and xylanase, and housekeeping genes such as β tubulin and *URA3*. 
Dot blot and virtual Northern analysis showed different levels of upregulation of various *C. minitans* genes during sclerotial colonization. Characterization of some potentially key genes obtained through all three procedures has now begun. Through complementation of a specific REMI mutant with the cloned wild type gene we have been able to restore pathogenicity and demonstrate that the pathogenicity less phenotype was the result of a defined insertional mutagenesis event. GFP reporter technology has been developed to permit temporal and spatial investigations into pathogenicity expression and is being used to underpin our investigations into gene silencing. Currently results indicate a role for various genes associated with overcoming stress during the sclerotial mycoparasitism process by *C. minitans*.

**Acknowledgements**

We would like to thank the BBSRC and the EU through project “2E BCAs in crops” for their financial support.

**References**


Trichoderma harzianum T39 activity against Plasmopara viticola

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Abstract: Several strains of Trichoderma harzianum have the ability to protect crops from various diseases and were developed up to commercial plant protection products. T. harzianum strain T39 (TRICHODEX) is particular interesting as it was indicated to reduce several diseases. However, to optimize control potential of the product it is relevant to understand its mode of action, the efficacy in controlling various stages of the disease and the potential interaction through the host plant. We focused on the mode of action of T. harzianum T39 in controlling Plasmopara viticola, the causal agent of grapevine downy mildew. In vitro and in planta tests were performed to test the effect of T. harzianum T39 on sporangia and oospores germination and on artificial infections under greenhouse controlled conditions and on natural infections in field experiments. T. harzianum T39 was not able to prevent oospore germination. No visible effect on sporangia and no prevention of zoospore release were detected, but the leaf disk assays highlighted the ability of the biocontrol agent to prevent P. viticola infections. Under greenhouse conditions, on artificially inoculated plants, T. harzianum T39 is more effective when applied before inoculation and more than one time. This and the absence of direct toxic effect on zoospores can indicate the induction of resistance in the plant. Certain disease control was reached in field trials, however, at a lower level compared to the standard reference (copper).

Key words: alternative products, biocontrol agent, copper, plant extracts

Introduction

A wide range of diseases is currently controlled in viticulture by chemical pesticide, but concerns for environmental pollution and the development of pesticide resistances require the substitution of chemicals with environmental friendly means of control such as biocontrol agents (BCAs). Easily isolated from soil, decaying wood, plant surfaces and plant organic material, microorganisms belonging to the genus Trichoderma represent promising BCAs. Their potential as antagonists is due to the production of antibiotic substances, to the induction of defence responses in plants and to the ability to antagonize and parasitize phytopathogenic fungi (Howell, 2003). Strains of T. harzianum, T. virens, and T. viride are the most widely studied microorganisms with potential control of fungal of plant diseases; some of them already became commercial products to be used for plant protection and growth enhancement in a wide range of crops. Trichoderma harzianum strain T39, developed at the Volcani Center in Israel and marketed as TRICHODEX, was primarily commercialized for the grape market (Elad, 1994) to control Botrytis diseases in greenhouse crops in Israel and other countries (Elad, 1995). The modes of action of T. harzianum T39 are competition for nutrients, interference with production of pathogenicity enzymes by the infecting pathogen (Elad, 1996) and induced resistance (De Meyer et al., 1998).

The objective of this research was to evaluate the efficacy of TRICHODEX against downy mildew caused by oomycete Plasmopara viticola.
Material and methods

Bioccontrol agent and pathogen
In the following experiments T. harzianum T39 (TRICHODEX, Makhteshim Ltd., Israel) was used at dosage 4 g/l. Suspension of P. viticola sporangia was prepared by washing fresh sporulating lesions on leaves with distilled water. The sporangia suspension was adjusted to $10^5$ conidia/ml. The direct inhibitory effect of P. viticola sporangia germination (zoospores release) was evaluated. 500 µl of sporangia suspension were mixed with 500 µl of TRICHODEX suspension (1:1). After 15 minutes any possible change of sporangia morphology was assessed and the percentage of germinated sporangia was counted under light microscope and compared to P. viticola sporangia water suspension (untreated control).

Leaf disk assays
The possible inhibition of P. viticola infection by T. harzianum T39 was studied using grapevine leaf disks treated with TRICHODEX/P. viticola sporangia (1:1) suspension and incubated in humidity chambers at 20°C. As control, leaf disks were treated only with the sporangia suspension/water (untreated control). At the end of the incubation period the presence of sporulation was evaluated. Leaf disks were put in contact with TRICHODEX by floating them on the BCA suspension for five minutes, artificially infected by spraying a water suspension of P. viticola sporangia and incubated over-night in humidity chambers at 20°C. Control leaf disks were treated with water before inoculation. At the end of the incubation period the reduction of sporulation compared to the untreated control was determined according to the formula: $(GUC-GT)*100/GUC$, where GUC is the number of germinated sporangia in the untreated control and GT is its number in the treated control.

Oospore germination assay
The oospore germination inhibitory effect of TRICHODEX was evaluated using the leaf disk germination test described by Hill (1998). Infected leaves containing oospores, were collected in August 2003 from an untreated vineyard, air dried and blended adding distilled water to obtain a homogeneous mass. A sample of 60-70 g of leaf mass was mixed with 20 ml of TRICHODEX suspension. The mixture was buried in soil and let to over-winter. Untreated blended leaf material was buried, under the same condition, and used as standard for oospore germination rate in spring. From March 2004, disk tests were carried out weekly on the untreated mass to monitoring oospore germination. TRICHODEX-treated leaf mass samples were evaluated when the germination of untreated oospores occurred in less than 24 h. A control agent is considered to be able to prevent oospore germination when the germination did not occur in the treated sample, but it is present in the untreated control.

Greenhouse and field trial
Efficacy trials were carried out under controlled greenhouse conditions (20°C, 70±10% RH), using potted plants of the susceptible cultivar (Pinot Gris). Five plants (replicates) were used in each treatment. The plants were sprayed with TRICHODEX once (1X), twice (2X) or tree times (3X) with intervals respectively of 1, 3 and 6 days before P. viticola artificial inoculation. Adopted standards were water (untreated control) and copper hydroxide (DC Flow, Dal Cin Gildo, 0.5 g/l Cu$^{2+}$) sprayed 6 h before inoculation. Fresh sporangia of P. viticola were used as inoculum and plants were incubated overnight in darkness at 80% RH and 20°C. Ten days after artificial inoculation, severity (percentage of infected leaf area) and incidence (percentage of infected leaves) were assessed on all leaves. ANOVA and Tukey’s test were applied on arcsin$\sqrt{p/100}$ transformed data.

The field trial was carried out in an experimental organic vineyard in 2004 in Rovereto, Italy, on the cultivar Cabernet Sauvignon. The statistical design used was fully randomized
blocks with 4 replicates. TRICHODEX 400 g/hl and standard copper treatments (Kocide 2000, Du Pont De Nemours, 50 g/hl Cu²⁺) were used with a volume spray of 12 hl/hectare.

Results and discussion

Effect of *T. harzianum* T39 on *P. viticola* development
The observation of 50 sporangia had shown no alteration in sporangia morphology or dimension. The number of germinated sporangia treated with TRICHODEX (42/50) did not differ with the number of germinate sporangia in the untreated control (45/50). In leaf disks assay sporulation was observed on all leaf disks not treated with TRICHODEX (controls). No sporulation was observed on disks treated with TRICHODEX or with mixed suspension (and *P. viticola*).

Inhibition of oospore germination
The germination rate of oospores preventively treated with TRICHODEX was identical to the untreated samples. This *T. harzianum* T39 did not affect the formation and maturation of pathogen oospores suggests that and that it was not able to prevent or to delay germination of over-wintered oospores in natural conditions.

Control of disease under greenhouse and field condition
Compared to the untreated plants, a partial control of *P. viticola* was achieved with TRICHODEX applied three times before pathogen inoculation (Fig. 1). Two or a single treatment with TRICHODEX reduced the disease to a lower extent, however significantly compared to the untreated.

![Figure 1](image)

Figure 1. Downy mildew symptom severity on leaves of grapevine in greenhouse trials treated 1, 2 or 3 times (6, 3 days and 24 hours prior *P. viticola* artificial inoculation). Standard was a single copper hydroxide (6 hours prior inoculation). Different letters mean significant difference among treatments (ANOVA and Tukey’s test at \( P<0.05 \)).

Under controlled greenhouse conditions, on artificially inoculated plants, *T. harzianum* T39 is more effective when applied before inoculation and more than once. Field trials showed that a certain control of the disease can be reached, even if it was not able to reach the same efficacy as copper (Table 1). The absence of direct toxic effect on zoospores, but the
inhibition of infection on leaf disks, greenhouse controlled experiments and the reduction of disease obtained in the field, suggest that *T. harzianum* T39 does not directly kill *P. viticola*, but could be involved in the activation of plant resistance mechanism.

Plants were weekly treated (from beginning of May until half of August) and *P. viticola* severity and incidence on leaves and bunches were evaluated on 20/07/05, (three weeks before harvesting). Numbers with a letter in common are not significantly different according to Tukey test (*P*<0.05).

Table 1. Efficacy trial done in Rovereto (Italy) in 2004

<table>
<thead>
<tr>
<th>Products</th>
<th>Leaves</th>
<th></th>
<th>Bunches</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severity (%)</td>
<td>Incidence (%)</td>
<td>Severity (%)</td>
<td>Incidence (%)</td>
</tr>
<tr>
<td>Kocide 2000</td>
<td>0 a</td>
<td>0 a</td>
<td>4.7 a</td>
<td>54.5 a</td>
</tr>
<tr>
<td>Trichodex</td>
<td>11.1 b</td>
<td>70 b</td>
<td>30.1 b</td>
<td>82 b</td>
</tr>
<tr>
<td>Untreated</td>
<td>58.8 c</td>
<td>100 c</td>
<td>76.7 c</td>
<td>100 c</td>
</tr>
</tbody>
</table>

### Acknowledgements

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### References


Simultaneous disruption of two exo-β-1,3-glucanase genes of *Pichia anomala* significantly reduced the biological control efficiency against *Botrytis cinerea* and *Penicillium expansum* on apples

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**Abstract:** *Pichia anomala* (strain K), antagonistic yeast against *Botrytis cinerea* and *Penicillium expansum* on apples, may constitute an effective solution to be included in an integrated pest management programme aiming at reducing the environmental depredation caused by synthetic fungicides.

During a first molecular analysis of the modes of action, lytic enzymes were studied by cloning and sequencing of two genes coding for exo-β-1,3-glucanases (PAEXG1, encoding anchored Paexg1p and PAEXG2, encoding secreted Paexg2p). Separated inactivation of both genes didn’t affect the biological control properties of strain K despite previous biochemical results suggesting an opposite conclusion.

A selection marker recycling strategy (URA3-Blaster) was recently adapted to *P. anomala* strain K, allowing multiple disruptions with the sole *URA3* marker gene. This new molecular tool led to the simultaneous inactivation of both *PAEXG1* and *PAEXG2* genes into a single strain. The biocontrol efficiency, against *B. cinerea* and *P. expansum*, of the resulting new mutated strains was significantly affected after application on wounded apples. For the first time in the field of biological control, glucanase-mutated strains of yeast offered a lower level of protection as compared to the level of parental strains. Furthermore, the difference of protective level between glucanase-mutated strains and wild strain was significantly influenced by the yeast inoculum concentration and physiological stage of the fruit. The overall results underlined the complexity of the antagonistic relationship established within the host-antagonist-pathogen system.
Ultrastructural changes in *Sclerotinia sclerotiorum* sclerotia treated with *Berberis vulgaris* plant extract

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Abstract: *Berberis vulgaris* hydroalcoholic plant extract was obtained from stem bark, was analysed regarding the berberine content and was tested, in different concentrations, against the germination and growth of *Sclerotinia sclerotiorum* fungus on nutritive medium. In minimum fungicidal concentration, *Berberis vulgaris* plant extract, caused important ultrastructural changes of hyphae’s and plasmic membrane’s cell wall, of cytoplasmic content, etc., in the external and internal zone of sclerotia and caused their loss of germination capacity.

Key words: antifungal action, fungal colony, micrographs, transmission electron microscopy

Introduction

It is known that *Berberis asiatica* (Bhandari et al., 2000), *Berberis heterophylla* (Freile et al., 2003), *Berberis aetnensis* (Musumeci et al., 2003), etc., plant extracts exhibit inhibitory activity against some bacteria and fungi. In our study, we analyzed the action of *Berberis vulgaris* plant extract on the ultrastructural characteristics of *Sclerotinia sclerotiorum* fungus.

Material and methods

*B. vulgaris* plant extract was obtained from stem bark, "Berberidis cortex", by repercolation with 70 % ethylic alcohol (Ionescu-Stoian & Savopol, 1977) and was standardized to 1 % alkaloids, appreciated in berberine (Deliu et al., 1994). The quantification of berberine in the plant extract was done by high performance liquid chromatography method coupled with mass spectrometry (LC/MS/MS), compared to commercial berberine (Wu et al., 2005).

The activity of *B. vulgaris* plant extract on in vitro germination and growth of *Sclerotinia sclerotiorum* fungus, isolated from carrot, was studied by the agar dilution method. Malt agar nutritive medium (Atlas, 2004) from Petri plates (70 mm in diameter) was inoculated in the central point with one black sclerotium of *S. sclerotiorum* fungus, and the colonies were incubated at 22°C. The inhibitory effect of plant extract was appreciated, compared to standard berberine and control, 5 days after inoculation.
The percentage of mycelium growth inhibition (P) was calculated for each concentration of the plant extract and/or standard berberine, by the formula $P = \frac{(C-T)}{C} \times 100$, where $C =$ the diameter of the control colony; $T =$ the diameter of the treated colony, according to literature (Nidiry & Babu, 2005). The results were statistically analyzed by ANOVA and were considered significant at $P<0.001$.

Control and treated $S. \text{sclerotiorum}$ sclerotia with $B. \text{vulgaris}$ plant extract, in minimum fungicidal concentration (MFC), for 3 hours, were embedded in Vestopal 310 polyester wax and were sectioned with an LKB III ultramicrotome. A methodology previously described (Hayat, 2000) was used for ultrastructural studies by means of Jeol JEM 1010 transmission electron microscope.

**Results and discussion**

A quantity of 0.5668 mg berberine/ml was determined in $B. \text{vulgaris}$ plant extract (Fig. 1), by LC/MS/MS (Wu et al., 2005), which emphasized that berberine is the main alkaloid.

![Figure 1](image1.png)

**Figure 1.** Chromatograms for berberine in *Berberis vulgaris* plant extract: MS/MS (left) and UV at 343 nm (right) signals

The inhibitory action of $B. \text{vulgaris}$ plant extract and commercial berberine against $S. \text{sclerotiorum}$ fungus was proportional to the concentration in the nutritive medium. The plant extract had a more powerful inhibitory action than Fluka berberine, at the same concentration. The MFC of $B. \text{vulgaris}$ plant extract was recorded at 450 µl/ml, when mycelium growth inhibition of $S. \text{sclerotiorum}$ fungus was 100%. The MFC for Fluka berberine was 625 µl/ml (Table 1). The results were statistically significant ($P<0.001$) and confirmed the more powerful inhibitory action of *Berberis* total plant extracts, compared to some alkaloids of their content. Thus, methanolic and aqueous *Berberis asiatica* plant extracts had a more powerful antimicrobial activity against some microorganisms than berberine, the main alkaloid (Bhandari et al., 2000). These results can be explained by the complex content of $B. \text{vulgaris}$ plant extract, which contains, besides berberine, some other alkaloids, such as berbamine, oxyacanthine, magnoflorine, berberubine, etc. (Tămaș, 1999).

Transmission electron micrographs showed that $B. \text{vulgaris}$ extract caused significant ultrastructural changes in $S. \text{sclerotiorum}$ sclerotia compared to the control (Fig. 2). The micrographs from the internal zone of $S. \text{sclerotiorum}$ control sclerotia showed the hyphae components (cell wall, lipid bodies, mitochondria, nucleus, plasmalemma, and vacuoles)
connected by glucan (Şesan & Crişan, 1998). B. vulgaris plant extract caused irreversible ultrastructural changes in sclerotia hyphae from the internal zone, as well as the alteration of cytoplasmatic content, the rarefaction of cell wall, etc. The precipitation of the entire cytoplasmic content and the destruction of organelles and nucleus led to the loss of viability and germination capacity of sclerotia, after 3 hours of treatment with plant extract in MFC.

The functional relation between the cell wall and the cytoplasmic content in treated sclerotia was destroyed; therefore a less electrondense band was formed. The action of B. vulgaris plant extract abolishes forever cell wall’s barrier function, and the possibility to activate the enzymes bound to the cell wall, revealed by different studies (Isaac, 1992).

Table 1. The antifungal activity of Berberis vulgaris total hydroalcoholic plant extract and standard berberine against the mycelial growth of Sclerotinia sclerotiorum

<table>
<thead>
<tr>
<th>Concentration in the nutritive medium (µl/ml)</th>
<th>Colony’s diameter (mm), after 5 days</th>
<th>P (%)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
<td>100</td>
<td>300</td>
<td>50</td>
<td>52</td>
</tr>
<tr>
<td>200</td>
<td>450</td>
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</tr>
<tr>
<td>400</td>
<td>600</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>450</td>
<td>625</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

A = Berberis vulgaris plant extract; B = Fluka berberine; C = control; P = mycelial growth inhibition; - = absent

Figure 2. Transmission electron micrographs showing ultrastructural changes in Sclerotinia sclerotiorum sclerotia (internal zone) treated with Berberis vulgaris plant extract in minimum fungicidal concentration (right), compared to the control (left). Cy = cytoplasm; CW = cell wall; G = glucan; L = lipid bodies; M = mitochondria; N = nucleus; P = plasmalemma; S = periplasmic space; V = vacuoles.
The analysis of *B. vulgaris* plant extract activity in MFC (450 µl/ml) demonstrates the fungicidal effect of this preparation and justifies the recommendation to use it for *in vivo* biological control of *S. sclerotiorum*.

**Acknowledgements**

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**References**


Biocontrol of *Rhizoctonia solani* in tomato with *Trichoderma harzianum* mutants

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Abstract: Biocontrol of *Rhizoctonia solani* in glasshouse tomatoes was analysed using *Trichoderma harzianum* mutants. These were obtained from wild strains of *T. harzianum* (previously selected for their good biocontrol activity), after treatment with N-methyl-N-nitro-N-nitrosoguanidine (NG) or UV light A (320 nm) or C (256 nm). Pellets containing different *Trichoderma* strains and selected mutants (1.7 g pellet/L soil) were applied to a soil previously inoculated with *R. solani* AG 4 at transplant. Controls without *Trichoderma* and with added fungicide were compared with each wild strain and mutant. Evaluations considered canker formation, root growth, mortality and fresh and dry weight of tomato plants of cvs. 92.95 and Góndola. Also the persistence in soil under storage conditions at 22 and 5°C was evaluated. Results showed that mutants controlled better *R. solani* than their corresponding wild strains. Th 12 A10.1, Th 11 A 80.1 and NG 7 were the best mutants for control of *R. solani* in cv. 92.95; while in the cv. Góndola were not observed statistical differences in the different strains. In general these results indicate that the mutants mentioned above are more efficient than their corresponding wild types for the control of *R. solani* under high pressure of disease in glasshouse conditions, and have a good persistence in the soil under storage conditions at 22 and 5°C. Experiments under greenhouse conditions are being carried out.

Key words: *Lycopersicon esculentum*, *Phytophthora nicotianae*, *Pyrenoachaeta lycopersici*

Introduction

Fungi species of the *Trichoderma* genus are among the most used bioantagonists for control of plant diseases (Papavizas et al., 1982; Rey et al., 2000). To strengthen the positive effects of these bioantagonists, strategies have been used to increase their enzyme activities (quitinases, glucanases, and proteases), and strains have been obtained with enzymatic activities far superior to those of wild genotypes (Pérez & Morales, 2003). The objective of this research was to evaluate the degree of control of *Rhizoctonia solani* using mutant strains of *Trichoderma harzianum* under semi controlled conditions, and to determine their survival in the soil at two storing temperatures.

Material and methods

Effectiveness of *Trichoderma harzianum* mutants for control of *Rhizoctonia solani* on tomato seedlings

Cvs. 92.95 and Góndola tomato seedlings were planted on 2.3 L pots with disinfected soil and inoculated later with 21 g inoculum per pot, equivalent to 215 thousand colony forming units per gram of soil (CFU/g soil) (Santander, 2001) of *R. solani* AG 4 618 and with the best
bioantagonists for in vitro control of R. solani (Arias, 2005) obtained from strains of Trichoderma harzianum (Table 1). Treatments (Table 2) were compared with controls run with R. solani 618 and with R. solani 618 + Pencycuron 0.15 mL/pot. Plant mortality, canker formation at stem base, root development, aerial dry weight and fresh root weight (Fig. 1) were evaluated at the 4–5th fruit cluster. All the experiments were done with four replications.

**Survival level of Trichoderma spp. in the soil under two storage temperatures**

Glass vial flasks with 5 mL sterile soil were inoculated with 9 mg sodium alginate pellets containing each strain of Trichoderma, equivalent to the dosage used in the first bioassay. The vials were stored at 22 and 5°C. 1-g samples were recovered from the vial flasks at days 90, 120, and 180 from the beginning of the experiment to isolate inoculum, which were sowed extensively on a medium selective for T. harzianum (Williams et al., 2003).

| Table 1. Trichoderma harzianum strains selected as treatments* |
|---------------------------------|----------------|----------------|
| Wild strains | Mutants | Mutagenic agent |
| Th 650 | Th650-NG 7^1 | N-methyl-N-nitro-N-nitrosoguanidine |
| Th 11 | Th 11C 40.1^2 | UVlight-C (256 nm) |
| Th 12 | Th 11A 80.1^2 | UVlight-A (320 nm) |

*Applied as sodium alginate pellets (Besoain et al., 2004) at dosages of 1.7 g pellets/L soil (116,000-1,000,000 CFU/g pellet).

**Results and discussion**

**Efficacy of mutants to control Rhizoctonia solani in tomato seedlings**

The level of cancrosis was higher in cv 92.95 than in cv. Góndola (Fig. 1). The best results were obtained after treatment with strains Th 11A 80.1, Th 12A 10.1, and Th650-NG 7. These results allow understanding the levels of mortality, where the mutants exerted good control of R. solani and resulted in null mortality of tomato plants, except with strain Th 11C 40.1, where 20% mortality was registered (Fig. 1). T0 resulted in 100% mortality, followed by wild genotypes Th 12 and Th 650, with 80 and 60% mortality, respectively, both below the control treatment with fungicide (T1), which along with Th11 showed only 20% mortality.

In cv. Góndola, there were no statistical differences between treatments in cancrosis and root development, which explains the lower mortality compared to cv. 92.95. However, 40% plant mortality occurred in treatments T0 and Th 11A 80.1, while the control with pencycuron treatment (T1), showed again 20% mortality as in the Th 650 wild genotype. The remaining treatments showed no mortality.

Mutants Th 12 A 10.1 and Th 11A 80.1 obtained the highest yield when evaluating the yield of dry matter in cv. 92.95 (Table 2), although they were statistically different only from treatments T0 and Th 12. The large difference between mutant strain Th 12A 10.1 and its wild strain Th 12, which had one of the smallest yields is noticeable. When analyzing root fresh weight, again treatments T0 and Th 12 presented the least weight according to the levels of plant mortality reached in these treatments (Fig. 1). The lower levels of mortality of cv. Góndola plants allow understanding the absence of differences found between treatments for all parameters measured of biomass yield, contrasting with results for cv. 92.95.
Survival of strains of Trichoderma harzianum in soil
At 90, 120, and 180 days under storage conditions, T. harzianum strains maintained at 22°C increased their initial population levels, which were statistically different and much greater in all wild strains (Th 12, Th 11 y Th 650) except in the strain Th 11 C 40.1. At 5°C, all the strains increased their initial population (Table 3). In general, it may be concluded that mutants are more efficient than their corresponding wild types for the control of R. solani under high pressure of disease in glasshouse conditions, and that they also have a good persistence in the soil under storage conditions at 22 and 5°C.

Table 2. Effectivity of T. harzianum mutants in cvs. 92.95 and Góndola tomato plants

<table>
<thead>
<tr>
<th>Treatments</th>
<th>cv. 92.95</th>
<th>cv. Góndola</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerial dry weight (g)</td>
<td>Root fresh weight (g)</td>
</tr>
<tr>
<td>Th 12A 10.1</td>
<td>9.60 a</td>
<td>3.72 a</td>
</tr>
<tr>
<td>Th 11A 80.1</td>
<td>9.20 ab</td>
<td>4.52 a</td>
</tr>
<tr>
<td>NG 7</td>
<td>7.98 ab</td>
<td>3.46 a</td>
</tr>
<tr>
<td>Th 11</td>
<td>5.38 abc</td>
<td>3.68 a</td>
</tr>
<tr>
<td>T1</td>
<td>8.20 ab</td>
<td>1.36 ab</td>
</tr>
<tr>
<td>Th 11C 40.1</td>
<td>7.10 ab</td>
<td>4.08 a</td>
</tr>
<tr>
<td>Th 650</td>
<td>5.02 abc</td>
<td>1.40 ab</td>
</tr>
<tr>
<td>T0</td>
<td>3.76 bc</td>
<td>0.02 b</td>
</tr>
<tr>
<td>Th 12</td>
<td>0.70 c</td>
<td>0.28 b</td>
</tr>
</tbody>
</table>

Means in a column with the same letter, indicate no statistical differences, according to Tukey multiple comparison tests at P≤0.05. 1/. T0 (only R. solani 618). 2/. T1 (R. solani + Pencycuron (0.15 mL/pot).
Table 3. CFU/g soil x1000 with the different strains of *Trichoderma harzianum* recovered from the vials maintained at 5 and 22°C

<table>
<thead>
<tr>
<th>T</th>
<th>Day</th>
<th>Mutants</th>
<th>Wild strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>22°C</td>
<td></td>
<td>Mutants</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>Th 12A 10.1</td>
<td>Th 11A 80.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52.5 a</td>
<td>87 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>230 c</td>
<td>112.4 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>545 b</td>
<td>385 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>78.3 b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>Th 11C 40.1</td>
<td>Th 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95.6 a</td>
<td>90 ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>187 a</td>
<td>1738 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>756 b</td>
<td>1322 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>165 a</td>
<td>210 a</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>Th 650</td>
<td>Ng 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>108.0 a</td>
<td>1158 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>292 a</td>
<td>1372 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>756 b</td>
<td>1116 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>556 a</td>
<td></td>
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<tr>
<td>5°C</td>
<td></td>
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<tr>
<td></td>
<td>90</td>
<td>Th 12</td>
<td>Th 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65.0 b</td>
<td>110 c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>420 b</td>
<td>155 c</td>
</tr>
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<td></td>
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<td>130 b</td>
<td>415 b</td>
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<td></td>
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<td>81 b</td>
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<tr>
<td></td>
<td>120</td>
<td>Th 11</td>
<td>Th 650</td>
</tr>
<tr>
<td></td>
<td></td>
<td>310.0 a</td>
<td>270 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>810 a</td>
<td>370 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>390 ab</td>
<td>610 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>475 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>Th 12</td>
<td>Th 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>514.0 a</td>
<td>498 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>876 a</td>
<td>712 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>572 a</td>
<td>1116 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>556 a</td>
<td></td>
</tr>
</tbody>
</table>

Means of four replicates in a column with the same letter indicate no statistical differences, according to Tukey multiple comparison tests at $P \leq 0.05$.

Acknowledgments

Research project FONDECYT 1040531-04.

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Perez, L.M. & Morales, M.P. 2003: Obtention of *Trichoderma harzianum* Th$_{650}$ mutants with antagonistic capacity against *Fusarium oxysporum*. In: Resúmenes XIII Congreso de la Sociedad Chilena de Fitopatología, Marbella, Chile.


The plant growth promoting and plant strengthening effects of *Trichoderma harzianum* strain T- (TRIANUM) on horticultural crops

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**Abstract:** Since 1999 Koppert Biological Systems has investigated the effect of *Trichoderma harzianum* strain T-22 (TRIANUM) on overall plant development in many different crops, in various substrates, under different climatic conditions. Research was carried out under (semi-) field conditions, sometimes in the presence of (naturally occurring or inoculated) soil-borne diseases.

Plant growth promoting and plant strengthening effects were obtained in a wide range of crops in the Netherlands, France, Spain and the United Kingdom. Plants, treated with TRIANUM showed a better developed root system (chrysanthemum, Calistephus, tomato), more flower buds (Kalanchoe, Lysianthus, Saintpaulia, Poinsettia), earlier flowering (Kalanchoe), a more uniform crop (Ficus, Dizygotheca, Chrysanthemum), a higher plant weight (Kalanchoe, Lysianthus, lettuce) and a higher yield (tomato, cucumber and bean). As TRIANUM increases the resistance of plants to stress (caused by diseases or sub-optimal conditions), plant strengthening effects like reduced plant loss and better plant development under stress conditions were observed in different crops (Cosmos, Impatiens, Viola and raspberry). In this paper an overview is given of the results obtained in ornamental crops, grown in organic substrates, in various countries. Also the performance of TRIANUM in other crops and substrates, like rockwool, and different application methods, are discussed.
Antifungal activity of secondary metabolites from the biocontroller *Beauveria bassiana* (Bals.) Vuillemin on orange coffee rust *Hemileia vastatrix*

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Abstract: *Beauveria bassiana* is an entomopathogenic fungus used for biological control of several insect pests. Part of this biological activity is attributed to the action of some toxins such as Beauvericin (BEA) and Basianin. In the present research work, the antimicrobial activity of these metabolites was evaluated by using the Coffee Rust as model. The results show significant statistical differences in average germination between treatments and controls (H20 and culture media), as well as between treatment doses. A linear increase of inhibiting effect on rust germination as response to increasing in toxins concentration was observed. In addition, significant statistical differences were observed among the treatments in relation with the control, when the disease curves constructed on infection indices were evaluated. This opens a potential area for the use of these metabolites as complementary tools for disease management, not only in coffee but also in other crops.

Key words: beauvericin, biological control, entomopathogeneous, mycotoxin

Introduction

*Beauveria bassiana* is the insect biological control most commonly used in our country. This fungus produces a great variety of secondary metabolites (mycotoxin Beauvericin, Tenellin, Bassianin, Bassianolide, Beauveriolide, Oosporein and others) along of its infection process, which contribute to its success in the insect pest control. In spite of the fact that this entomopathogeneus fungus is currently used for pest control, it could also be used for the management of some economically important diseases (Gemma et al., 1985). This duplicity of action is due in great part from its capacity to produce a broad spectrum of secondary metabolites; such as toxins, which are bioactives peptides with high potential to be used in both agricultural and medicinal application. These molecules may be additional tools for vegetal protection (Arboleda et al., 2004). Coffee is attacked by different plagues and diseases affecting seriously the production of the grain. The orange coffee rust, which is produced by *H. vastatrix*, is considered today in our country as an important disease; because of its broad geographical distribution and the economical damage that it could produce.

Material and methods

**Biological material**

*B. bassiana* isolates Bb 9024, Bb 9010, Bb 9205 and Bb 9001 were obtained from the Microorganism collection at the National Coffee Research Center, CENICAFE, Colombia. Standard solutions of Beauvericin (Sigma) and Bassianin (received from Dr. Leo C. Vining...
Dalhousie University. Canada) were used in the present work. Uredospores of the *H. vastatrix* were collected from leaves of *Coffea arabica* var. Caturra which were growing in field.

**Liquid extracts from isolates of *B. bassiana***
Flasks containing 100 ml of Sabouraud liquid medium were inoculated with each isolate to a final concentration of $1.4 \times 10^7$ conidia/ml; all of them were maintained in special room during 9 days. After this, the mycelium was separated by vacuum filtration, centrifugation and the supernatant was collected to perform the various tests.

**Germination test**
To inoculate the coffee leaves, uredospores of *H. vastatrix* were prepared in solutions containing 1, 2, 5, 10, 15 and 20% of liquid extract from isolates of *B. bassiana*, Beauvericin and Bassianin. Liquid medium without inoculation and uredospore suspension in water were used as controls. The uredospore germination was observed after 12 hours.

**Evaluation of defense protein expression**
Ten coffee plants were sprayed with liquid extract from Bb 9024 (High BEA production) and with Beauvericin pure (0.1 mg/ml). The controls used were only culture media and water. Protein contents were estimated from coffee leaves that were collected from plants maintained in greenhouse during 5, 10 and 15 after aspersion. In this case, a buffer 20 mM Tris-HCl pH 8/0 containing 1 mM EDTA, 5 mM DTT, 0.1 mM PMSF and 1% PVP was used to perform the extraction. Lipoxygenases, chitinases and glucanases expression was detected by using polyclonal antibodies.

**Effect of liquid extracts from *B. bassiana* on Coffee Orange Rust in greenhouse conditions**
The Coffee plants were sprayed with liquid extracts from isolates Bb 9010, Bb 9205 and Bb 9001 corresponding to high, middle and low BEA production, and liquid medium and water as controls. In all cases a high (100% extract) and low (50% extract) dose was used. 24 hours after, the plants were inoculated with uredospores ($6.5 \times 10^4$ u/ml) and maintained under special conditions during three days and finally they were put in greenhouse with the goal of analyzing the development of disease based on a specific affection scale (Leguizamón, 1983). The same experiment was realized in two different weather conditions: June – August (dry time) and November – January (Rainy time).

Curves of disease were constructed calculating the infection indices based on the following formula: If = $\sum n(g)/(N \times 7)$; where n corresponding to lesions in each grade (g), N is the inoculation total number (16) and 7 is the maximum grade in the Leguizamón’s scale. The Infection Period (PI) is the time when 75% of sites inoculated had lesions < grade 1; and Latency period (PL) is the time when 75% of sites inoculated show sporulation (grades ≥ 4). Under a completely randomized design, with ANOVA and Tukey (5%) this experiment was analyzed.

**Results**
Our results have shown an inhibition effect in all treatments that were tested. This inhibition was directly proportional to the extract concentration used (Table 1). By using Dot Blot methodology three enzymes related with defense were observed in all treatments. This detection was higher in plants sprayed with pure BEA than in other ones. Highest levels and severity of the lesions, corresponding to controls were observed for the two different weather conditions evaluated. However, although in less proportion, symptoms of the disease were observed in the treatments too; but in many cases at a lower level. In both experiments, it was observed that liquid extracts from *B. bassiana* 9205 (High) and Bb 9001 (Low), showed lower infection levels during all evaluation period than control treatment (Liquid medium) which did not interfere with the development of the disease (Fig. 1).
Table 1. Germination of *H. vastatrix* uredospore after treatment with different concentrations of liquid extracts from isolates of *B. bassiana* fungus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Germination (%)</th>
<th>Standard Deviation</th>
<th>CV</th>
<th>Test per treatments</th>
<th>Test among treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0</td>
<td>82,20</td>
<td>1,53</td>
<td>0,018</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Medium Culture</td>
<td>0</td>
<td>84,44</td>
<td>3,24</td>
<td>0,042</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>55,22</td>
<td>0,655</td>
<td>0,011</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>53,43</td>
<td>1,784</td>
<td>0,033</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Bb 9010</td>
<td>5</td>
<td>46,72</td>
<td>1,270</td>
<td>0,027</td>
<td>C</td>
<td>b</td>
</tr>
<tr>
<td>High BEA</td>
<td>10</td>
<td>37,54</td>
<td>1,016</td>
<td>0,027</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>Production</td>
<td>15</td>
<td>32,23</td>
<td>3,157</td>
<td>0,097</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>17,63</td>
<td>1,313</td>
<td>0,074</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>50,32</td>
<td>2,607</td>
<td>0,051</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>54,84</td>
<td>1,878</td>
<td>0,034</td>
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<td></td>
</tr>
<tr>
<td>Bb 9205</td>
<td>5</td>
<td>40,05</td>
<td>2,946</td>
<td>0,073</td>
<td>C</td>
<td>b</td>
</tr>
<tr>
<td>Middle BEA</td>
<td>10</td>
<td>34,08</td>
<td>3,748</td>
<td>0,109</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>Production</td>
<td>15</td>
<td>29,52</td>
<td>4,175</td>
<td>0,141</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20,84</td>
<td>3,986</td>
<td>0,191</td>
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<tr>
<td></td>
<td>1</td>
<td>68,31</td>
<td>1,852</td>
<td>0,027</td>
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<tr>
<td></td>
<td>2</td>
<td>66,54</td>
<td>4,985</td>
<td>0,074</td>
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<tr>
<td>Bb 9001</td>
<td>5</td>
<td>55,95</td>
<td>0,831</td>
<td>0,014</td>
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<td>b</td>
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<tr>
<td>Low BEA</td>
<td>10</td>
<td>46,97</td>
<td>2,032</td>
<td>0,043</td>
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<td></td>
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<tr>
<td>Production</td>
<td>15</td>
<td>41,26</td>
<td>1,511</td>
<td>0,036</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>34,80</td>
<td>4,788</td>
<td>0,137</td>
<td>e</td>
<td></td>
</tr>
</tbody>
</table>

* Values with the same letter do not have significative statistical difference. Duncan 5%.

Discussion

Secondary metabolites from *B. bassiana* seriously affect the development of the *H. vastatrix* fungus growing on coffee plants. This could be explained by two different mechanisms: a protective effect trough inhibition of uredospores germination and a inductive effect on defensive plant response, which is characterized by the protein activation related with plant defense allowing a significative reduction of the disease at the end of the treatment.

In spite of the fact that many studies with *B. bassiana* toxins has been performed testing its biological activity on different insect pests, it was showed that secondary compounds from different entomopathogenic fungi had antimicrobial activity too (Nilanonta et al., 2000; Gemma et al., 1985).

*B. bassiana* produces a very interesting quantity of secondary compounds, which could be tested and used for control of different microorganisms causing important diseases on economically important crops. Looking for new active biomolecules against not only insect pest, but also against destructive microorganisms, could contribute to a sustainable agriculture keeping its natural biodiversity with a consequent economic benefice and the environment conservation.
Figure 1. Curves of disease based in the Infection Indices corresponding to all treatments evaluating during November – January (Rainy-time) (A) and June – August (dry-time) (B)

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References


Molecular study of the yeast *Pichia anomala* strain K by inactivation of genes using the *URA*-Blaster technique

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**Abstract:** The yeast *Pichia anomala* strain K has been selected for its antagonistic activity against *Botrytis cinerea*, one of the most important pathogen of apples in postharvest environment. As a biocontrol agent, this yeast constitutes an interesting alternative to chemical treatments. In order to improve its antagonistic activity, the modes of action of the yeast have to be studied more deeply.

A recycling strategy for selection marker (*URA3*-Blaster) was recently adapted to *Pichia anomala* and successfully applied for successive inactivation of 2 genes coding for Exo-ß-1,3-glucanases (Friel et al.). Ten genes are potentially involved in the antagonist relation of *P. anomala* have been identified by cDNA-AFLP (Massart et al., 2006). Those genes were all overexpressed when the yeast was grown on a medium supplemented with cell walls of *B. cinerea*. Regarding to their potential function, 5 genes have been selected and are currently separate inactivated. The biocontrol efficiency of new mutated strains will be evaluated during *in vivo* experiments and compared with the parental strain K. Results will be discussed.

**References**
Friel D., Jijakli M.H., Vandenbol M., Gomes Pessoa M.N., Separate and simultaneous disruptions of two Exo-ß-1,3-glucanase genes decrease the biocontrol efficiency of *Pichia anomala* (strain K) against *Botrytis cinerea* on apples, submitted to MPMI.

Fluorescent microscopic studies in the interactions of *Pichia anomala* and *Aspergillus flavus*

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**Abstract:** *Pichia anomala* Strain WRL-076 has been used as a biocontrol agent to reduce aflatoxin, growth and spore production of *Aspergillus flavus*. The objective of this study was to probe the antagonistic effect of the yeast, *P. anomala* against *A. flavus* by using a vital fluorescent stain, FUN-1. Yeast and fungi were inoculated into a 250 ml-flask containing 50 ml potato dextrose broth (PDB) at yeast to fungus (Y : F) ratios of 1:1, 5:1, 10:1, 30:1 and 50:1. Hyphae of *A. flavus* were harvested and stained by a fluorescent compound, FUN-1 and then viewed through a Leica DMRB epifluorescence microscope. Metabolically active *A. flavus* hyphae accumulated red fluorescence in vacuoles, while hyphae that were inhibited by *P. anomala* stained green. The result indicates that the yeast might inhibit the ATP system of *A. flavus*, which resulted in significant reduction in fungal biomass.

**Key words:** aflatoxin, biocontrol mechanism yeast

**Introduction**

Several yeast species have been used commercially as biocontrol agents for post-harvest or pre-harvest control of fruit diseases (Janisiewicz & Korsten, 2002; Spadaro & Gullino, 2004). Three strains of the yeast species, *P. anomala*, have been demonstrated to control storage mold in small grains (Peterson & Schnurer, 1998), to reduce fruit rot in apple (Jijakli & Lepoivre, 1998), in grape vine (Masih et al., 2002) as well as to prevent aflatoxin and ochratoxin production by *A. flavus* and *Penicillium roqueforti* (Peterson & Schnurer, 1995; Hua et al., 1999; Hua, 2004, 2006). It is important to understand the mechanisms by which antagonistic yeasts control the target fungus because the resulting information can facilitate the development of effective methods for formulation, application and commercial registration.

The FUN-1 fluorescent stain is a membrane-permeate, halogenated cyanine compound that binds to nucleic acids. FUN-1 has been used to study antifungal agents against several species of *Aspergillus* species (Lass-Florl et al., 2001; Marr et al., 2001; Balajee et al., 2002). Biochemical processing of the dye by metabolically active fungal hyphae yielded cylindrical intra-vacuolar structures (CIVS) that were markedly red shifted in fluorescent emission and spectrally distinct from the nucleic acid bound form of the dye resulting in green (Millard et al., 1997). In this study FUN-1 was applied to study mechanism of biological control by yeast and to visualize the physiological response of *A. flavus* to *P. anomala* WRL-076.
Material and methods

Microorganisms and chemicals
The toxigenic strain Aspergillus flavus Strain 18-3, used in this study was maintained on potato-dextrose agar (PDA, Difco, Detroit, MI). Fresh cultures of P. anomala were propagated on PDA as well. FUN 1 [2-chloro-4-(2,3 dihydro-3-methyl (benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide] was purchased from Molecular Probes Inc. (Eugene, OR); Tween-80, glucose and HEEPS (N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid) were purchased from Sigma Chemical Co. St. Louis, MO).

Experimental design
Yeast and fungi were inoculated into a 250 ml-flask containing 50 ml potato dextrose broth (PDB). For inoculation into PDB, fungal spores and yeast cells were re-suspended in 0.05% Tween 80 and in sterile distilled water respectively, and then counted in a Beckman Coulter Multisizer II (Beckman Coulter, Fullerton, CA, USA). Each flask was inoculated with 1 ml of fungal spore suspension at 10^6 ml^-1 and the final concentration of the yeast cells in the medium was adjusted accordingly to the yeast to fungus (Y : F) ratios of 1:1, 5:1, 10:1, 30:1, and 50:1. The flasks were incubated at 28°C for 48 h with gentle shaking. Hyphae of A. flavus were harvested and separated from the yeast cells by filtering through the Cellector tissue sieve with 38.1 µm filter. As a control, fungal spores were inoculated into 50 ml PDB. Dead hyphae were produced by boiling hyphae for 30 min. Both living and dead fungal hyphae were used as the controls in the staining studies.

Fluorescent staining
The fungal hyphae harvested on the sieve screen were gently rinsed with sterile deionized water before transferred to a 1.5 ml-microfuge tube containing 1 ml sterile deionized water. The hyphae suspension was then centrifuged at 10,000 rpm in an Eppendorf microfuge for 5 minutes. The supernatant was removed and FUN 1 in HEPES buffer was added to the hyphae and incubated at 28°C in the dark for 30 minutes. The stained hyphae were spotted on a slide and viewed through a fluorescein filter in a Leica DMRB epifluorescence microscope. The fluorescein filter enabled the visualization of both green fluorescent hyphae and red fluorescent CIVS. Images were captured with a Sony DKC-5000 digital color camera.

Influence of yeast on A. flavus fungal biomass
Yeast cells and fungal spores were inoculated at the ratios of (yeast : fungus) of 1:1, 5:1, 10:1 and 50:1 into 50 ml of PD broth and incubated at 28°C for 48 h with gentle shaking. A. flavus was grown in PD broth in the absence of yeast to be used as the control. Fungal hyphae were collected by using a Cellector tissue sieve fitted with a 38.1 µm filter. The hyphae were rinsed with distilled water, transferred to a pre-weighed filter paper and then dried in an oven at 65°C over-night. The samples of dried hyphae were weighed using a balance.

Results and discussion

Antagonistic effect of P. anomala on A. flavus
The optimal concentration for staining of live A. flavus was 10 µmol l^-1 FUN1 in HEPES buffer (PH 7.0). Thus viable A. flavus hyphae stained with FUN-1 showed intense red fluorescence in the vacuole when visualized by epi-fluorescence microscopy (Fig. 1A). In contrast, heat killed A. flavus stained with FUN1 showed only bright green fluorescent hyphae devoid of red vacuoles (Fig. 1D). As the inoculation ratios of yeast cells to spores increased, the FUN1 accumulated in the vacuoles decreased significantly. All hyphae of A. flavus grown in PD broth without P. anomala were metabolically active, as evidenced by the red
fluorescent intra-vacuolar CIVS within the hyphae and by the relatively low and diffuse green fluorescence of the hyphae as shown in Fig. 1A. Hyphae of *A. flavus* grown with the yeast *P. anomala* in a ratio of 1:30, displayed few red fluorescent vesicles while a high amount of the hyphae had bright green fluorescence that is characteristic of low metabolic activity (Fig. 1B), a small number of hyphae remained viable, as determined by FUN1 staining. Most hyphae of *A. flavus* grown in the presence of *P. anomala* at a ratio of 1:50, lacked red fluorescent vesicles and had bright green fluorescence (Fig. 1C); their appearance was similar to heat-killed *A. flavus* (Fig. 1D).

**Reduction of fungal biomass**

The dry weights of *A. flavus* mycelia grown in PDB at the yeast to fungal spore ratios of 0:1, 1:1, 5:1, 10:1 and 50:1 were 57.5 (sd, 4.1), 16.9 (sd, 2.6), 10.9 (sd, 1.1), 3.4 (sd, 0.6) and 0.3 (sd, 0.26) mg, respectively (Fig. 2). The data suggested that the yeast inhibited the growth of *A. flavus* by 70.6% in the 1:1 yeast to fungus samples as compared to the control in which yeast was not present. When the ratio increased to 50:1, the dry mycelia weight decreased by 99.5%, which corresponded to a 192-fold decrease. ANOVA analysis of fungal biomass of *A. flavus* indicated the antagonistic effect of yeast was significant (Fig. 2).

![Figure 1](image1.png)

**Figure 1.** Epifluorescence micrographs of live, yeast treated, and heat killed *Aspergillus flavus* stained with FUN1 viability stain. Details of the experiments were described in the methods above.
Figure 2. Effect of *P. anomala* on *A. flavus* fungal biomass when both were inoculated in cultural media at increasing ratios of yeast cells to fungal conidia. Details of the experiments were described in the methods above.

A major mechanism of yeast biocontrol agents is their effectiveness in competing for nutrients (Janisiewicz & Korsten, 2002; Spadaro & Gullino, 2004). Production of cell wall degrading enzyme, exo-β-1,3 glucanase was implicated in *P. anomala* strain K (Jijakli & Lepoivre, 1998; Grevesse et al., 2003). Our preliminary experiments indicate the induction of PaEXG2 gene expression of *P. anomala* WRL-076 by *A. flavus* when co-culturing in the same medium. (data not shown). This enzyme probably caused damage on the hyphae of *A. flavus* and affects ATP system resulting in significant reduction fungal growth.

References


Study of the modes of action of two biocontrol agents Z1 and ZH2

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Abstract: Penicillium italicum represents, together with P. digitatum, Geotrichum candidum and Phytophthora citrophthora, one of the most important postharvest diseases on citrus. Lost in storage conditions can reach 60% in the absence of treatments. Beside the classical chemical treatments, hazardous for consumers and environment, there is a need for alternative control methods. Biological control, susceptible to complete an integrated management program constitutes, in this context, an interesting approach.

Two biocontrol agents (Z1, yeast strain and Zh2, bacterial strain) were isolated from Citrus fruits surface and selected for their effectiveness against P. italicum. To go further into the development of a biopesticide, the modes of action of both strains must be understood. The aim of this study was to evaluate the possibility of competition for nutrients and the antibiotic production of each antagonistic strain.

Competition for nutrients between both antagonists and P. italicum was in vitro tested according to Janisiewicz et al. (2000). Pathogen (in the cylinder) and BCAs (in the well) were separated by filter allowing the diffusion of nutrients only. Spore germination of the pathogen was quantified under microscope, after growing in medium supplemented with increasing amount (0.1, 0.5, 1, 5, 10 and 15%) of natural orange juice.

Strain Z1 inhibited the spore germination, as compared to control, for the low juice concentration (up to 5%). The addition of fresh juice after antagonist removal allowed the restoration of the germination. This suggests the possible implication of competition for nutrients in the biocontrol activity of Z1. The experiments were repeated with bacterial strain ZH2 but didn’t underline any competition symptom.

The antibiotic production was in vitro tested for the two strains, by co-inoculation with P. italicum on Petri plates. Two media (NYDA and YEPD for Z1, PCA and PDA for ZH2) and 4 co-inoculation distances (1, 2, 3 and 4 cm) were tested for each strain. A growth inhibition of the pathogen was observed after co-inoculation with ZH2 on PCA medium. Nevertheless, a co-inoculation on agar-supplemented orange juice medium didn’t confirm the results, suggesting a high influence of the medium on the antibiotic production. No antibiosis symptom was detected for strain Z1 against P. italicum.
Effect of the biological control strain *Serratia plymuthica* HRO-C48 on verticillium wilt of olive trees cv. Arbequina

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Abstract: Integration of biological control measures to protect olive planting material produced by nurseries could help managing Verticillium wilt in olive. Therefore, the application of *S. plymuthica* HRO-C48 to suppress *Verticillium dahliae* in seven-month old olive plants cv. Arbequina was investigated. The method of infestation with the pathogen, either by soil inoculation or by root dipping, determined the effect of the biocontrol agent. Using soil inoculation, HRO-C48 treatment reduced the disease severity. In addition, a statistically significant plant growth promoting effect was observed for HRO-C48 in non-pathogen stressed plants.

Key words: Olea europea, Verticillium dahliae

Introduction

Verticillium wilt, caused by *Verticillium dahliae*, is one of the most serious diseases affecting olive trees worldwide and is responsible for severe yield losses and plant death (Jiménez-Díaz et al., 1998). Until recently, effective control of *V. dahliae* was achieved, among other chemical control measures, by fumigating the soil with methyl bromide. This and other related substances show highly toxic effects to many non-target organisms as well as additional negative influence on the world climate. Therefore, in developed countries the application of methyl bromide was ultimately banned in 2005. The management of Verticillium wilt disease should be based on an integrated strategy, which involves primarily the choice of planting sites with low inoculum densities and increasing certainty that pathogen-free plants come from the nurseries. The beneficial rhizosphere microbiota can be enhanced for increased plant health by the introduction of beneficial microorganisms, and therefore offers an environmentally friendly alternative to control verticillium wilt. The beneficial Gram-negative bacterium *Serratia plymuthica* strain HRO-C48 was isolated from the rhizosphere of oilseed rape and selected as a biocontrol agent according to the following criteria: (a) high antifungal activity against fungal pathogens, e.g. *V. dahliae* and *Phytophthora cactorum* *in vitro*, (b) production of the plant growth hormone indole-3-acetic acid, (c) relative harmlessness to human health and the environment; and (d) low level of antibiotic resistance (Berg, 2000). The successful application of *S. plymuthica* HRO-C48 was shown for strawberries (Kurze et al., 2001). Here, the bacteria were able to colonize the rhizosphere to avoid an infection with *Verticillium dahliae*, and to enhance fruit yield. A product called RhizoStar® was developed for commercial strawberry production (Berg et al., 1999). Mercado-Blanco et al. (2004) have described the first-time employment of antagonistic
bacteria to protect olive nursery-plants against *V. dahliae*. In planting material of the highly susceptible olive cultivar ‘Picual’, the introduction of root-associated pseudomonads resulted in suppression of the defoliating (D) pathotype of *V. dahliae*. Engaging a similar experimental design, but using the susceptible cultivar ‘Arbequina’, the capacity of *S. plymuthica* HRO-C48 to protect olive planting stocks against *V. dahliae* was investigated.

**Material and methods**

**Preparation of bacterial and fungal inocula**

The inoculum of *Serratia plymuthica* HRO-C48 (DSMZ 12502) was grown on NA at 30°C for two days, scraped from the medium with sterile Drigalski spatula, and suspended in 10 mM MgSO₄·7H₂O. Bacterial suspensions were centrifuged twice in 10 mM MgSO₄·7H₂O to remove residual metabolites (10,500× g, 20 min) and re-suspended in 10 mM MgSO₄·7H₂O solution. Bacterial concentration in the suspension were adjusted to log₁₀ 8.7 CFU ml⁻¹. Monoconidial *V. dahliae* isolate V138I used in this study is a representative of the highly virulent, D patho-type originated from diseased cotton plants (Culture collection Instituto Agricultura Sostenible, CSIC, Córdoba, Spain). For bioassays, inoculum consisted of conidia from cultures in potato-dextrose broth (PDB) incubated in the dark for seven days at 24°C and 125 rpm. Conidia were harvested by filtering liquid culture through a layer of sterile cheesecloth and inoculum concentration was adjusted to log₁₀ 7.3 conidia ml⁻¹ by dilution with sterile water.

**Bioassay experimental design**

The growth chamber trial was arranged in a two-stage setup. For the first 90-day stage, seven-month old, micropropagated olive plants cv. Arbequina, provided by Cotevisa (Valencia, Spain), were carefully uprooted from the substrate, their roots thoroughly washed in tap water, and dipped into a *S. plymuthica* HRO-C48 cells suspension for 10 min. For the control treatment, plants were dipped in 10 mM MgSO₄·7H₂O. Plants used for assessment of plant growth promotion (16 per treatment) were transplanted (one per pot) into clay pots with a diameter of 15 cm containing an autoclaved (twice on consecutive days) soil mixture (sand/loam, 2:1, v/v). For the biocontrol activity evaluation, 20 plants were grown in soil which was infested by thoroughly mixing 100 ml of a conidial suspension of *V. dahliae* V138I with 1.0 kg of soil mixture to obtain a final concentration of log₁₀ 6.3 conidia g⁻¹ soil. The experiment was conducted in a randomized block design. Plants were incubated in a controlled growth chamber at 23±1°C, 60 to 90% relative humidity, and received a 14 h photoperiod for three months. Plants were watered as needed, and fertilized weekly with a hydro-sol fertilizer 20-5-32+microelements (Haifa Chemicals, LTD, Haifa, Israel). During the 90 days, no symptoms of Verticillium wilt could be observed. Thereafter, plants, which were grown in infested soil, were again exposed to the pathogen in two different ways. For one hand, ten plants per treatment were uprooted and replanted into 20-cm-diameter clay pots filled with autoclaved sand/loam mixture (2:1, v/v) infested with log₁₀ 7.0 conidia g⁻¹. On the other hand, soil-free root bales of another ten plants each treatment were trimmed using sterile scissors, dipped into conidial suspension (log₁₀ 7.0 conidia ml⁻¹), and transplanted into 20-cm-diameter containing sterile sand/loam mixture. Finally, four plants each treatment from the plant growth-promoting assay were also replanted into 20-cm-diameter containing sterile sand/loam mixture after they were uprooted. Olives were arranged in a randomized block design and were grown for another 79 days under same conditions described before.

**Assessment of disease development and plant growth**

Disease reaction was assessed by the percentage and severity of symptoms on a 0-4 rating scale according to the percentage of affected leaves and twigs (0 = no symptoms, 1 = 1–33%,
Data were subjected to analysis of variance. Percentage values were arcsine transformed \((Y/100)^0.5\) before analyses. Data on disease severity were used to calculate the following: (i) a disease severity index \((DII)\) determined as \(DII = (\sum Si \times Ni)/(4 \times Nt)\), where \(Si\) is the symptoms severity, \(Ni\) is the number of plants with \(Si\) symptoms, and \(Nt\) is the total number of plants; and (ii) the standardised area under disease progress curve of \(DII\) plotted over time (AUDPC) calculated as described previously (Mercado-Blanco et al., 2004). Treatment means were compared with those of the control using the Dunnett’s test at \(P = 0.05\). For assessing plant growth promotion, total plant length, calculated by summing length of stem and lateral branches, was scored. Initial measurements were done after transplanting treated olives. The plant growth promotion experiment was terminated after 90 days, and total plant length was recorded again to calculate the relative plant growth. Four plants were grown another 79 days after replanting into a new pot and measured once again.

Results and discussion

*S. plymuthica* HRO-C48 was shown to be able to colonize the rhizosphere as well the endorhiza of olive plants until 168 days post-inoculation (data not shown). After HRO-C48 treatment and planting in *V. dahliae*-infested soil, no symptoms caused by the D isolate V138I evolved in olive plants during 90 days. Thus, the experimental plants were once more exposed to the pathogen. Fig. 1 illustrates the course of the disease development from the moment of the second inoculation. For *S. plymuthica* HRO-C48 bacterized plants, a suppression of the Verticillium wilt was found when *V. dahliae* was applied by soil inoculation rather than by root dipping. Using soil infestation, all plants developed symptoms characteristic to those caused by the D pathotype. In control plants, Verticillium wilt developed by 32.9 days reaching a final \(DII\) of 0.64 and an AUDPC of 27.8. Bacterization with HRO-C48 reduced the \(DII\) by 23.4%, and AUDPC by 40.6% (Fig. 1A). Results obtained by immersing olive roots into conidial suspension were different to those of soil infestation. In non-bacterized control plants, first symptoms appeared by 25.7 days post-inoculation reaching a final \(DII\) of 0.71 and AUDPC of 31.2. The disease development in *S. plymuthica*-treated plants started by 24.9 days, and the final \(DII\) (0.81) as well the AUDPC (38.6) were slightly higher compared to the control plants (Fig. 1B). The ability of the bacteria to delay the development of symptoms caused by the D pathotype of *V. dahliae* depended on the inoculation procedure. Compared to the soil infestation method, it is apparent that the rhizosphere of plants dipped into the conidial suspension was disturbed by this procedure. It could be speculated that strain HRO-C48 cells, which operates from the outer surface, were washed off by the immersion and were thus unable to prevent the root from penetration by fungal hyphae. Incorporation of the fungal inoculum into the growth substrate represents the more realistic form of *Verticillium* infestation. In that case, the rhizosphere and its bacterial community remain intact. The putative ‘protective shield’ formed by *S. plymuthica* was able to impede the fungal attack.

The ability to promote plant growth in the absence of the pathogen was determined by scoring stem and branch length after 90 and 169 days (Fig. 2). No statistically significant differences between the bacterial-treated and non-bacterized (control) plants were observed after 90 days. The relative length growth of the control plants amounted to 313±79% compared to 339±71% of plants inoculated with HRO-C48. After 169 days, however, the bacterization of olive roots with strain HRO-C48 resulted in a statistically significant increase of stem and branch growth of 837±78% on average, compared to the control plants (657±54%). Woody plants such as olives are known to grow relatively slowly. Accordingly, growth effects originating from bacterial activity ought to manifest in a long-term scale.
Hence, experiments to evaluate plant growth promotion should be of duration longer than five months. In conclusion, under conditions similar to those in the olive-nursery industry, the effect of *S. plymuthica* HRO-C48 on disease development and plant growth was demonstrated. Importantly, even three months after root bacterization, HRO-C48 was shown to provide some degree of disease protection. It can be suggested, that once HRO-C48 is introduced, the biocontrol agent may provide a long-term protection against soil-borne fungi, even when the plant is replanted into new, potentially infested soil.

![Figure 2](image1.png)

**Figure 2.** Effect of *S. plymuthica* HRO-C48 on the development of Verticillium wilt of olive cv. Arbequina. *Verticillium dahliae* was inoculated by either soil infestation (A) or root dipping. The disease intensity index (*DII*) ranging from 0-1 was calculated with data on incidence and severity of symptoms recorded at 7-days intervals.

![Figure 3](image2.png)

**Figure 3.** Plant growth promotion effect of *S. plymuthica* HRO-C48 on olive cv. Arbequina assessed by monitoring total plant length. Bars with same letters were not significantly different according to Fisher’s protected LSD test (*P* ≤ 0.05).

**Acknowledgements**

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References

Identification of genes involved in the production of the antibiotic 2-hexyl, 5-propyl resorcinol and its role in biocontrol

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Abstract: The white root rot disease of avocado (Persea americana) is caused by the fungi Rosellinia necatrix, and it is the most destructive disease of this crop in the Mediterranean area. Few approaches have been developed for its control, and recently, the use of rhizobacterial strains in biological control is being under study in order to improve the integrated management of the avocado crop. One bacterial strain, Pseudomonas fluorescens PCL1606, has been isolated from rhizosphere of healthy avocado trees and selected for its antagonistic ability against many soil-borne phytopathogenic fungi. Biocontrol experiments using the tomato/Fusarium and avocado/Rosellinia test systems showed that P. fluorescens PCL1606 has a high biocontrol ability.

The analysis of the antifungal compounds produced by P. fluorescens PCL1606 only detected the production of the antibiotic 2-hexyl, 5-propyl resorcinol (HPR), which could be unique responsible of the antagonistic activity of this bacterial strain. In order to study the role of HPR in biocontrol, derivative mutants impaired in the antagonistic activity were constructed by Tn5 mutagenesis. From approximately 7,000 mutants obtained, seven derivative strains defective in antagonistic activity have been selected and characterized. The Tn5 flanking regions were analyzed, revealing putative genes involved in the production of HPR. Biocontrol experiments on the experimental test systems, showed reduction of the protection levels when using these derivative mutants. The reduction in biocontrol activity was not complete, suggesting that more than one trait could be involved in the biocontrol activity of the P. fluorescens PCL1606 strain.

At the same time, the operon previously described in P. aurantiaca to be involved in the HPR biosynthesis (dar operon) have been detected in P. fluorescens PCL1606 DNA. Site-directed mutants on these genes also were constructed, and the obtained mutants showed reduction in the antagonistic activity against different fungi. The effect of such directed-site mutations on the biological control against R. necatrix is been studied. The disrupted genes generated by the two mutagenesis methods will be recovered from a phage library of P. fluorescens PCL1606, in order to perform complementation experiments and mapping of these genes.
Characterization of the role of \textit{luxS} in the fire blight pathogen \textit{Erwinia amylovora}

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**Abstract:** Fire blight, caused by \textit{Erwinia amylovora}, is among the most serious diseases of pome fruits and related Rosaceae. Biocontrol is being explored as an alternative to current control options of prevention and sanitation measures and, in the U.S.A., to antibiotic applications (mostly prohibited in Europe). Current biocontrol strains inhibit pathogen growth through antibiosis or competition. We are seeking novel approaches for biocontrol through reduction in virulence and environmental fitness by targeting gene regulatory systems. We investigate here the role of \textit{LuxS} in \textit{E. amylovora} in order to understand its role as putative producer of the autoinducer-2 (AI-2) signal and as metabolic enzyme in the activated methyl cycle (AMC), with the ultimate aim to elucidate its impact on bacterial pathogenicity. Our data show that the production of virulence factors, pathogenicity and the ecological competence of \textit{E. amylovora} is in fact slightly reduced in \textit{luxS} mutants, but that this is rather due to the importance of \textit{LuxS} in the metabolic pathway of the AMC, rather than to AI-2 production and quorum sensing.

**Introduction**

\textit{Erwinia amylovora}, is a gram-negative, enteric phytopathogen that is the causal agent of fire blight, a devastating disease of apples, pears and related rosaceous trees. As a member of the \textit{Enterobacteriaceae}, \textit{E. amylovora} is related to many important plant pathogens such as \textit{Erwinia carotovora}, \textit{Serratia marcescens} or \textit{Pantoea agglomerans}, and animal pathogens like \textit{Escherichia coli}, \textit{Yersinia pestis}, \textit{Salmonella typhimurium} or \textit{Shigella flexneri}. We show here that like these other gram-negative bacteria \textit{E. amylovora} features a \textit{luxS} gene, which displays a dual role in many bacterial cells: as metabolic enzyme in the activated methyl cycle (AMC) and in quorum sensing as producer of the AI-2 signal (Winzer et al., 2003).

The AMC is responsible for the generation of the major methyl donor S-adenosyl-L-methionine (SAM) and the recycling of methionine by detoxification of S-adenosyl-L-homocysteine (SAH). LuxS takes part in this cycle by salvaging the homocysteine moiety from the cycle intermediate S-ribosyl-homocysteine (SRH). As a by-product of this reaction, the direct AI-2 precursor (S)-4,5-dihydroxy-2,3-pentanedione (DPD) is formed.

In bacteria such as \textit{Vibrio harveyi} or \textit{Salmonella} sp. DPD is then transformed in the actual AI-2 extracellular signal (furanosyl borate diester), which is then detected via the LuxPQ two-component sensor kinase or Lsr system respectively. AI-2 is well described in animal pathogenic bacteria where it's crucial for the transition to pathogenic existence inside the host, but its role in plant pathogens is still unknown. Screening of potential biocontrol agents with AI-2 reporter strains may result in natural antagonists that quench quorum-sensing. On the other hand, phenotypes linked to the disruption of the AMC are known to display impaired growth and reduced environmental fitness. Therefore, the knowledge of the role of \textit{luxS} in plant pathogens may pave the way to new control strategies.
Methods and results

**Sequence analysis of the luxS gene in a global collection of E. amylovora strains**

An 843-bp fragment spanning the genes corB, luxS and gshA was sequenced in a collection of *E. amylovora* strains of worldwide origin. Sequences were obtained for 21 strains and representative luxS examples are available in the NCBI database (accession numbers DQ457094 and DQ457095). Sequence analysis indicated that luxS is extremely conserved (>99%) among genotypically diverse *E. amylovora* strains, pointing to an important role of LuxS in the life cycle of the pathogen. *In silico* analysis was performed to find other AI-2 related genes in *E. amylovora*, but no homologues of luxPQ genes (Neidtich et al., 2005) or the lsr operon (Xavier et al., 2005), encoding for AI-2 receptors in *V. harveyi* and *Salmonella* spp. respectively, were found in the *E. amylovora* genome.

**Development of luxS mutants of E. amylovora**

luxS mutants of *E. amylovora* strains FAW610 and CFBP1430 were obtained by inserting a kanamycin resistance cassette into the gene using double homologue recombination. Complementation of *E. amylovora* mutants CFBP1430 luxS and FAW610 luxS was performed by transforming the strains with plasmid pMF8805 carrying the intact *E. amylovora* luxS gene. Both cross-feeding assays (Bassler et al., 1997) and gene expression studies confirmed the complete knock-out of luxS transcription in the two mutants (Fig. 1) and the restoration of AI-2 production in the complemented strains (Table 1).

![Figure 1. mRNA expression of luxS gene in E. amylovora CFBP1430 wildtype (wt), mutant (mut) and complemented (cpl) monitored by reverse transcription PCR. (+) positive control containing CFBP1430 DNA, (-) negative control (H2O).](image)

Table 1. Induction of *V. harveyi* BB170 by *E. amylovora* in different culture media after 14h.

<table>
<thead>
<tr>
<th>Strain</th>
<th>AB</th>
<th>LB</th>
<th>Davis</th>
<th>hrp</th>
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</thead>
<tbody>
<tr>
<td><em>V. harveyi</em> BB170</td>
<td>100*</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CFBP1430</td>
<td>6.5</td>
<td>7.5</td>
<td>8.5</td>
<td>10.5</td>
</tr>
<tr>
<td>CFBP1430 luxS</td>
<td>bd</td>
<td>0.5</td>
<td>bd</td>
<td>0.5</td>
</tr>
<tr>
<td>CFBP1430 luxS/pMF8805</td>
<td>7</td>
<td>6.5</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>FAW610</td>
<td>4</td>
<td>6.5</td>
<td>6.5</td>
<td>9</td>
</tr>
<tr>
<td>FAW 610 luxS</td>
<td>bd</td>
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<tr>
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<td>7.5</td>
<td>6.5</td>
<td>9.5</td>
</tr>
</tbody>
</table>

*The level of BB170 activity in AB was normalized to 100%.

**Phenotypic characterization of luxS mutants under abiotic conditions**

The role of LuxS in phenotype and fitness was evaluated *in vitro*. Motility assays were performed with *E. amylovora* CFBP1430 and FAW610 strains and luxS derivatives on LB plates containing decreasing concentrations of agar. Co-cultivation (competition) experiments
were performed to observe the impact of medium composition on the growth and genetic expression of wild-type, mutant and complemented strains. No significant differences in motility/swarming were detected between the wild-type strains and their derivatives in media with a range of 0.3-0.8% agarose. Cultivation experiments showed that the mutant strains were severely impaired in growth in modified (sulfate-free) Davis minimal medium under sulfur limiting conditions, i.e. 50 \text{M} methionine (met) as sole S-source, but not in Davis minimal medium (DMM) where sulfate concentration (8.4 \text{mM}) is not a limiting factor (Fig. 2). This phenotype was not relieved under co-culture conditions suggesting that the observed effect was not of an extra-cellular signal nature, but of metabolic nature.

![Figure 2. Growth of FAW610 wildtype (wt), luxS mutant (mut), and complemented (cpl) strains in DMM or Davis minimal medium modified by replacing ammonium and magnesium sulfates with the corresponding chlorides and supplemented with $5 \times 10^{-4}$ M methionine (met).](image)

**Genetic expression in luxS mutants under abiotic conditions**

Genetic expression was evaluated by monitoring RNA transcription of \textit{luxS}, pathogenicity related \textit{hrpL} and \textit{dspA} genes, and several metabolic genes: \textit{srlD} (sorbitol metabolism), \textit{galE} (galactose metabolism), \textit{scrK} (fructose metabolism), and activated methyl cycle genes (\textit{metE}, \textit{metK}, \textit{metJ}, \textit{metR} and \textit{pfs}) under different conditions (e.g., high-low nutrient availability, sulfur limitation) using semi-quantitative RT-PCR. Constitutive \textit{rrs} gene encoding for 16S rRNA was used as internal control for sample normalization (Fig. 3). \textit{luxS} was most strongly expressed in minimal medium, a condition that may mimic the plant environment, and was slightly higher with cysteine (cys) than with methionine (met) as a sole S-source. The experiments performed indicate that the expression of critical pathogenicity genes \textit{hrpL} and \textit{dspA} are down-regulated in \textit{luxS} mutants entering the end of the exponential growth phase and in the stationary phase. In contrast, expression of the carbon metabolism genes \textit{galE} and \textit{srcK}, and the activated methyl cycle genes \textit{metE}, \textit{metK}, \textit{metJ} and \textit{metR} is upregulated in \textit{luxS} mutants. The expression of \textit{srlD} increased upon reaching the stationary phase, but was independent from \textit{luxS} or the available sulfur source, while \textit{pfs} was constantly under the detection limit for all the strains throughout the experiment. The expression of \textit{rrs} (16S-RNA) was confirmed to be constitutive throughout all growth phases.
Figure 3. Expression of different metabolic and pathogenicity-related genes in *E. amylovora* FAW610 wild-type (wt) and mutant (mut) monitored by semi-quantitative reverse transcription PCR. Cys or met were used at 50 μM as sole S-sources in modified DMM.

Figure 4. Apple flower colonization by *E. amylovora* FAW610 wildtype (wt), mutant (mut), and complemented (cpl) strains after 1-2 days in detached flower assays. Bacterial suspensions (10⁶ CFU ml⁻¹) were inoculated in the hypanthium and quantitative assessment of population growth was performed after two days by dilution plating on selective media.

**Effect of luxS mutation on E. amylovora flower colonization and virulence**

The role of *luxS* in apple flower colonization was studied by inoculating the primary infection court (stigmas/hypanthium) with bacterial suspensions (wild-type, mutant and complemented strain) and quantitative assessment of population growth after 1-2 days in detached flower assays. The effect of the *luxS* mutation on the pathogenicity of *E. amylovora* was investigated by inoculating shoots of ‘Golden Delicious’ apple seedlings and 2 year old trees in the quarantine greenhouse and by measuring lesion development over 2-3 weeks. Ability to colonize apple flowers was reduced by approximately 1 log in *luxS* mutants compared to wild-type or *luxS*-complemented strains. Results were slightly more exaggerated when mutants were co-inoculated with either the wild-type or complemented strains compared to when strains were inoculated individually (Fig. 4). Virulence of mutants was slightly reduced,
but was not abolished, assays in 8 weeks old seedlings as determined by reduction in lesion development and formation of bacterial ooze in seedling. Conversely, no significant reduction of virulence was obtained with 4 weeks old seedling, suggesting that the role of LuxS in virulence may depend on host physiological status (data not shown).

Conclusions

Our data show that the production of virulence factors, the pathogenicity and the ecological competence of *E. amylovora* is in fact slightly reduced in the luxS mutant. However, the low AI-2 levels detected (which may be explained by the spontaneous conversion of DPD to AI-2), the reduced performance of the mutant in minimal medium and in apple flowers, together with the alteration in the expression of the genes related to the methionine pathway, lead us to hypothesize that the importance of LuxS for *E. amylovora* lies more in its metabolic role in the AMC, rather than in AI-2 production and quorum sensing. Furthermore, the absence of known receptors for AI-2 signals (homologues of *luxPQ*, encoding the two-component sensor kinase of *V. harveyi*, or of *lsr*-operon of *Salmonella* spp.) strongly suggest that, even if *E. amylovora* is capable to produce low amounts of AI-2, it’s not able to detect this compound and hence to use this molecule as a extracellular signal for quorum sensing.

References


SyrTox project: mechanism of action of *Pseudomonas* spp. metabolites and their potentiality in the biocontrol

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Abstract: *Pseudomonas syringae* is a gram negative bacterium with a double identity: it is a plant pathogenic organism causing disease on many crops, but it is also a biocontrol agent of plant pathogens. Some secondary metabolites are responsible of both activities, known as lipodepsipeptides (LDPs), which are largely produced in culture. The project SyrTox proposes to clarify some of the mechanisms through which the bacterium exerts its antagonist activity and to use this knowledge to widen the field of application of the bacterium and its metabolites as biocontrol agents. A similar perspective exists also for *P. tolaasii* and *P. reactans*, which use LDPs to attack edible mushrooms and have similar antagonistic activity.

Key words: *Pseudomonas syringae, Pseudomonas tolaasii, Pseudomonas reactans, WLIP*

Details of activities

*Pseudomonas syringae* is a gram negative bacterium with a double identity: it is a plant pathogenic organism causing disease on many crops, but it is also a biocontrol agent of plant pathogens. Its application as antagonist in agriculture has reached the commercial level in many countries, USA included, but not yet in Italy. Responsible of both these activities are some secondary metabolites, known as lipodepsipeptides (LDPs), which are largely produced in culture. The SyrTox project is oriented toward clarifying some of the mechanisms through which the bacterium exerts its antagonist activity and the use of this knowledge to widen the field of application of the bacterium and its metabolites as biocontrol agents. A similar perspective exists also for *P. tolaasii* and *P. reactans*, which use LDPs to attack edible mushrooms and have similar antagonistic activity.

The project in particular is studying the biosynthesis and mechanism of action of the LDPs produced by the pseudomonades, so to gain a deeper knowledge of their antibiotic activity (Dalla Serra et al., 2004, 2005; Grgurina et al., 2005; Scaloni et al., 2004). Clarifying the main biosynthetic pathways (Fullone et al., 2005; Giovannini et al., 2005) could allow the production of structural analogues of the metabolites, with more interesting biological activity, and a better understanding of the structural implications. Another important aspect of this project is the development of convenient model systems to be used to select the most effective metabolites and to determine the best conditions of application. The models must be of laboratory size, with easy preparation and storage with guarantee of its availability at any
time. Another system should be reproducing a natural host/pathogen system and allow rapid and inexpensive testing. We are currently developing the protocols. Once LDPs of the desired characteristics are selected, their persistence in the treated fruits (pre- and post-harvest) and in the fruit products (juices, fruit salads, jams, distillations) will be carefully determined, to exclude the existence of any possible related risks.

Acknowledgement

This paper presents research done in SyrTox project funded by Fondo per la Ricerca, Autonomous Province of Trento.

References


Mechanism of action against *Plasmopara viticola* of the grapevine endophytic strain of the fungus *Alternaria alternata*

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**Abstract:** Endophytes are potentially good candidates for biocontrol. An endophytic strain of *Alternaria alternata* was isolated from a grapevine showing atypical and mild symptoms of *Plasmopara viticola*. Its culture broth applied on *Vitis vinifera* leaf disks artificially inoculated with *P. viticola* was shown to inhibit *P. viticola* sporulation. Ultrastructural alterations of *P. viticola* haustoria and mycelium in tissues treated with *A. alternata* broth before and after inoculation were observed. The aim of this work was to explore the biocontrol activity of *A. alternata* and its mechanism of action against *P. viticola* on grapevine. Leaf disks were treated with *A. alternata* culture broth prior to *P. viticola* artificial infections, contemporarily and after artificial inoculation. To understand if several applications can boost a resistance mechanism in the plant, *A. alternata* culture broth was also repeatedly applied before *P. viticola* inoculation. Untreated controls and copper treatments were included as standards. Half of the leaf disks were incubated to check for pathogen sporulation and the other half was used for relative quantification of *P. viticola* DNA by means of multiplex real-time quantitative polymerase chain reaction. Preventive treatments with *A. alternata* culture broth (three and one day before inoculation) and concurrent treatment did not inhibit infection and sporulation. When leaf disks were treated one day after inoculation with *P. viticola* no sporulation at all was observed. When *A. alternata* culture broth was applied several times before infection, sporulation was inhibited even if the pathogen was able to establish inside the tissue. The efficacy of *A. alternata* in inhibiting *P. viticola* after inoculation was confirmed also on plants under greenhouse controlled conditions. The results suggest a mode of action through an interaction of the plant with the metabolites produced by *A. alternata* rather than a mere direct toxic effect on *P. viticola*.

**Key words:** biocontrol, downy mildew

**Introduction**

Endophytes are potentially good candidates for biocontrol for several reasons: (i) they are extremely widespread colonizers of various plant species, (ii) they usually survive the whole plant life inside the host tissues often without any apparent negative effects, (iii) some of them are able to induce defence mechanisms in host plants, (iv) they are able to provide active plant protection occupying the micro-habitat of some pathogens, and (v) some of them can produce toxins against insects and growth hormones useful to the plant’s metabolism. An endophytic strain of *Alternaria alternata*, was isolated from a grapevine showing atypical and mild symptoms of *Plasmopara viticola*. Its culture broth applied on *Vitis vinifera* leaf disks was effective against *P. viticola* artificial infections completely inhibiting the sporulation. Ultrastructural analyses carried out to observe cellular interactions between *P. viticola* and *A. alternata* in the grapevine leaf tissue show that, even without close contact with *A. alternata*, the *P. viticola* mycelium has severe cytological alterations, such as the presence of enlarged vacuoles or vacuoles containing electron-dense precipitates (Musetti et al., 2006b). Haustoria
appear necrotic and irregularly shaped or enclose in deposits of callose-like substances. Three diketopiperazines: cyclo(L-phenylalanine-trans-4-hydroxy-L-proline), cyclo(L-leucine-trans-4-hydroxy-L-proline) and cyclo(L-alanine-trans-4-hydroxy-L-proline) produced by *A. alternata* in culture broth were isolated and characterised (Musetti et al., 2006a). A mixture of the three diketopiperazines was very efficacious in limiting *P. viticola* sporulation both on artificially inoculated grapevine leaf disks and greenhouse plants. A toxic action of diketopiperazines produced by *A. alternata* against *P. viticola* was therefore hypothesized (Musetti et al., 2006a).

The aim of this work was to explore the biocontrol activity of *A. alternata* and its mechanism of action against *P. viticola* on grapevine.

**Material and methods**

**Leaf disk assay**

Agar plugs with *A. alternata* mycelium, maintained on Potato Dextrose Agar (Sigma) at 4°C, were transferred to nutrient broth (Oxoid) and incubated with shaking for 5 days at 25°C. Grapevine plants of the susceptible cultivar Pinot Gris, having two shoots with four fully expanded leaves each, were used in the bioassays. Five plants (replicates) were used for each treatment. Plants were treated with *A. alternata* culture broth prior *P. viticola* artificial inoculation (3 and 1 day before), contemporarily (0 day) and one day after artificial inoculation. To understand if several applications can boost a resistance mechanism in the plant, *A. alternata* culture broth with cells was also applied with a two day interval, four times (5, 3, 1 and 0 days before inoculation), three times (3, 1 and 0 days), twice (1 and 0 days) and once at 0 day (contemporarily) (Table 1). Ten leaf disks for each replicate of each treatment were cut from leaves. The inoculation with *P. viticola* was done on 10 leaf disks randomly cut from each treated plant, by floating them on the *P. viticola* sporangia suspension (4.5×10⁵ sporangia/ml), over-night at 20°C. Half of the leaf disks were incubated at 20°C and saturating air humidity to check for pathogen sporulation and the other half was used for relative quantification of *P. viticola* DNA by means of multiplex real-time quantitative polymerase chain reaction. The treatment on day 0 was applied one hour before of inoculation. Copper hydroxide (Kocide 2000, DuPont) at a concentration of 0.5 g Cu²⁺/l and water sprayed plants were used as standard and untreated control. Five disks per treatment were kept in moist chambers until sporulation was seen on the untreated control. Sporulation was visually assessed on the leaf disks using a stereomicroscope.

**Table 1.** Application times (days prior and after *P. viticola* inoculation) of *Alternaria alternata* culture broth on grapevine leaves. “0” refers to *P. viticola* inoculation time.

<table>
<thead>
<tr>
<th>Days from <em>P. viticola</em> inoculation</th>
<th>-5</th>
<th>-3</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
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<tr>
<td>x</td>
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</table>
**Real-time PCR**

The remaining forty five leaf disks were transferred in multi-well plates for the DNA extraction and analysis. They were freeze-dried and the DNA was extracted with NucleoSpin Multi-96 Plant Kit (Macherey-Nagel, Duren, Germany). Instead of the lysis buffer supplied, CTAB was used and instead of one final elution of 180 µl, three elution of 60 µl were obtained. The DNA collected in the second elution was afterwards analysed with a quantitative real time polymerase chain reaction.

The protocol for DNA extraction and PCR as described by Valsesia et al. (2005) was followed. DNA of samples, pure *P. viticola* DNA, pure *V. vinifera* DNA and a no-DNA control template were amplified. Multiplex reaction containing 1x TaqMan Universal Master Mix, 250 nM *P. viticola* VIC-labelled probe (Giop P), 250 nM *V. vinifera* FAM-labelled probe (Res P), 900 nM *P. viticola* forward and reverse primers, 120 nM *V. vinifera* forward and reverse primers and 5 µl template DNA. Amplification were performed with the standard short cycling parameters (50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min). Results are shown as ratio of CT *V. vinifera* over CT *P. viticola* (infection coefficient). A high ratio (IC) between *P. viticola* DNA/ *V. vinifera* DNA indicated successful infection and tissue colonization by *P. viticola* and low control activity. Conversely, low IC indicated low *P. viticola* colonisation and good disease control.

**Greenhouse treatments**

Grapevine plants of the susceptible cultivar Pinot Gris, having two shoots with four fully expanded leaves each, were used in the bioassays. Five plants (replicates) for each treatment were sprayed with *A. alternata* culture broth and cells (prepared as described in the leaf disk assay) 6, 12, 24 and 48 hours after *P. viticola* inoculation (4.5×10^5 sporangia/ml). Disease severity and incidence was assessed on all leaves of each plant at the end of the incubation period (10 days). Copper hydroxide (Kocide 2000, DuPont), at a concentration of 0.5 g Cu^{2+}/l, and nutrient broth were used as standard and untreated control.

**Statistical analysis**

ANOVA and Fisher LSD test (*P*≤0.05) were used for statistical analysis of arcsin transformed data.

**Results and discussion**

Preventive treatments with *A. alternata* culture broth (three and one day before inoculation) and concurrent treatment did not inhibit infection and sporulation. When leaf disks were treated one day after inoculation with *P. viticola* no sporulation at all was observed and a low concentration of *P. viticola* DNA was present (Fig. 1, left). When *A. alternata* culture broth was applied several times before infection, sporulation was inhibited even if the pathogen was able to establish inside the tissue (Fig. 1, right).

No reduction of disease was seen with *A. alternata* treatments applied on plants 6 hours after *P. viticola* artificial inoculation, but treatments applied between 12 and 48 hours after inoculation were as effective in reducing the diseases as copper hydroxide (Fig. 2).

If applied before *P. viticola* inoculation *A. alternata* requires repeated treatments in order to be effective in reducing the disease. This effect seems to be due to an inhibition of sporulation instead of a reduction of the tissue colonization by the pathogen. The effect of *A. alternata* in disease suppression starts to be present with treatments applied 12 hours from inoculation, therefore, without any direct contact between *A. alternata* and *P. viticola*. *A. alternata* does not act as resistance inducer in the plant since the high content of *P. viticola* DNA demonstrate that leaf colonization by the pathogen is present, however, it is able to interact with the sporulation process, when several treatments are applied.
Figure 1. Infection coefficient (P. viticola DNA/V. vinifera DNA) and presence of Plasmopara viticola sporulation on grapevine leaf disks treated with Alternaria alternata 3 days, 1 day, at the same time and 1 day after P. viticola artificial inoculation (left) and with repeated applications of A. alternata before pathogen inoculation (4, 3, 2 and 1 time). Columns with the same letter are not significantly different with Fisher LDS test, \( P \leq 0.05 \).

Figure 2. Plasmopara viticola severity on grapevine plants treated with Alternaria alternata culture broth and cells 6, 12, 24 and 48 hours after artificial inoculation with a water suspension of the pathogen (4.5×10⁵ sporangia/ml). Assessments were carried out 10 days after inoculation. Columns with the same letter are not significantly different with Fisher LDS test, \( P \leq 0.05 \).

*A. alternata* produces pathogen-inhibiting metabolites belonging to the groups of diketopiperazines, which showed similar post-infection efficacy compared to *A. alternaria* cells applied with culture broth (Musetti et al., 2006a). The results suggest a mode of action...
through an interaction of the plant with the metabolites produced by *A. alternata* rather than a mere direct toxic effect on *P. viticola*.

If the anti-sporulating efficacy against *P. viticola* and its activity during the first stages of infection will be confirmed also in field experiments, *A. alternata* or its metabolites could be a new biological tool to be further developed for grapevine protection against downy mildew.

**Acknowledgements**

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**References**


Efficacy of *Pseudomonas syringae* lipodepsipeptides in inhibiting *Botrytis cinerea* on strawberry fruits

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**Abstract:** *Pseudomonas syringae* is a gram-negative bacterium that can act as a pathogen for several crops, but also as a biocontrol agent against some plant pathogens. These activities have been correlated to some secondary metabolites largely produced in culture by the bacterium, known as lipodepsipeptides (LDPs). A preliminary assay was carried out using *Botrytis cinerea* on bean-detached leaves that allowed determining the optimal concentration of LDPs. The efficacy of some selected LDPs, obtained after purification through HPLC, to inhibit *B. cinerea* was tested on strawberry fruits. LDPs were used at different concentrations mixed 1:1 with *B. cinerea* conidia suspension with a final concentration of 5×10⁴ conidia/ml. Small drops (10 µl) were placed on the intact surface of strawberry fruits. Incidence and severity were recorded during shelf life in order to verify the efficacy of the toxins at different concentrations against *B. cinerea* and to study their effects on living tissues. The LDPs were able to reduce disease compared to the inoculation with *B. cinerea* alone. However, all toxins showed phytotoxicity on strawberry tissues.

**Key words:** biocontrol, corpeptin, LDPs, syringopeptin, syringomycin, strawberry fruits

**Introduction**

*Pseudomonas syringae* is a gram-negative bacterium with a double identity: it is a causal agent of disease for many crops, but it can also act as a biocontrol agent against fungal and bacterial diseases of numerous cultivated plants and in post-harvest. Strains of *P. syringae* have been reported to inhibit *Penicillium expansum* and *Botrytis cinerea* on pears (Sugar & Spotts, 1999), *Monilina fruticola* and *Rhizopus stolonifer* on peaches (Zhou et al., 1999), *P. digitatum* on citrus fruit, *B. cinerea* on grape (Cirvilleri et al., 2000) and *P. expansum* on apples (Conway et al., 1999). It is also an agent inducing resistance against *Plasmopara viticola* and *Erisiphe necator* on grape (Kassemeyer et al., 1998). Among several metabolites that *P. syringae* produces, peptidic secondary metabolites, known as lipodepsipeptides (LDPs), recovered from bacterium cultures (Ballio et al., 1991, 1994). LDPs can directly interact with microbiological membranes, their principal target, causing alteration of the membrane permeability (Dalla Serra et al., 1999). *P. syringae* LDPs proved to be quite effective in the growth inhibition of a wide array of fungi and bacteria (Lavermicocca et al., 1997; Fogliano et al., 2002). The sensitivity of the target organisms to the various *Pseudomonas* toxins has been found to be very different (Iacobellis et al., 1992).

Toxins were identified by mass spectrometry. The purification and the relative quantification were carried out using an HPLC method described in the literature (Ballio et al., 1994). Their biological effects were measured on natural systems in order to verify the
antagonism of selected LDPs towards pathogens. In this work we evaluated their efficacy to inhibit grey mould on the host/pathogen binomial *B. cinerea*/*strawberry*. *B. cinerea* is one of the most important diseases of strawberry (Elad et al., 2004).

**Material and methods**

**Toxin dilutions**

Serial dilutions of the 4 toxins syringomycin E (SRE), syringopeptin 22A and 25A (SP22A and SP25A) and corpeptin A (CPA) were prepared starting from mother solutions of 1 mg/ml.

**Inoculum preparation**

*B. cinerea* conidia were collected in water + 0.01% Tween80 from two weeks old well conidiating colonies on Petri dishes. After centrifugation and re-suspension in distilled water, conidia were counted and adjusted to the concentration of $1 \times 10^5$/ml supplemented with 0.1% glucose and 0.1% KH$_2$PO$_4$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Final concentration (mg/ml)</th>
<th>Incidence (%)</th>
<th>Severity (%)</th>
<th>Number of brown spots/drop</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRE 0.1</td>
<td>10.0 ab</td>
<td>7.5 abc</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>c-SRE 0.1</td>
<td>15.0 abc</td>
<td>4.0 ab</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>SP22A 0.1</td>
<td>20.0 abc</td>
<td>13.0 abc</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>SP22A 0.01</td>
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<td>2.5 ab</td>
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</tr>
<tr>
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<td>15.0 abc</td>
<td>7.5 abc</td>
<td>22</td>
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</tr>
<tr>
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<td>15.0 abc</td>
<td>13.8 abc</td>
<td>23</td>
<td></td>
</tr>
<tr>
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<td>15.5 bc</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>CPA 0.1</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>18</td>
<td></td>
</tr>
<tr>
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<td>15.0 abc</td>
<td>12.5 abc</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>CPA 0.001</td>
<td>15.0 abc</td>
<td>5.5 abc</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>c-CPA 0.1</td>
<td>15.0 abc</td>
<td>7.5 abc</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td><em>B. cinerea</em> 0</td>
<td>40.0 c</td>
<td>18.5 c</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Treatments indicated with c- were not inoculated with *B. cinerea*. All other treatments were inoculated with the toxin application with a final concentration: $5 \times 10^4$ conidia/ml.

Means followed by the same letter do not differ to each other according to LSD test at $P \leq 0.05$.

*Averages of 4 replicates of 5 fruits per treatment.*
Efficacy of toxins against B. cinerea on strawberry fruits
Commercial strawberry fruits not treated against B. cinerea and produced by APA Sant’Orsola (Italy) were used. Four replicates of five fruits placed in boxes per each treatment were used in a fully randomised block design. Each toxin dilution was mixed with conidia suspension (1:1) except for the control treatments, which only contained each toxin at the highest concentration (0.1 mg/ml) without conidia (1:1 in water), to allow the estimation of possible phytotoxicity. Three drops (10 µl) of the mixture were placed on the intact surface of each strawberry. Brown spots development and disease severity and incidence were assessed on each fruit. Data were analysed with ANOVA and means separated by Fisher LSD test with $P \leq 0.05$.

Results and discussion

The different toxins caused both phytotoxicity on strawberry fruits and inhibition of B. cinerea (Table 1). All the treatments with toxins alone induced small brown spots on the fruit skin where the drops were placed. All four toxins reduced the development of B. cinerea to a variable extent depending on the concentration and toxin. The treatments CPA-0.1, SP25A-0.001, SP22A-0.01, SRE-0.1 and cSRE significantly reduced B. cinerea incidence, while CPA-0.1, SP25A-0.001, SP22A-0.01, cSP25A and cSRE significantly reduced B. cinerea severity. The application of the toxins at the highest concentration induced the development of some B. cinerea infections even without artificial inoculation. This is the result of the heavy lesions caused by the toxin on the fruit skin, which allowed the penetration of some B. cinerea naturally present on the fruits.

The application of the toxins led to different results related to the different toxins and to their different concentrations, showing that each LDP needs concentration optimization in order to reach the highest efficacy in disease control and the lowest phytotoxicity on fruits, which can increase B. cinerea infections due to the caused lesions on fruit skin.

Acknowledgement

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References


Multiple effects of *Trichoderma* spp. applied to sugar beet towards soil-borne pathogens

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**Abstract:** The effectiveness of *Trichoderma* as biocontrol agent against a number of plant pathogens is reported to be based on several mechanisms, such as antibiosis, mycoparasitism, induction of defence responses and other adjunct mechanisms, such as growth promotion. Previous studies carried out both under controlled condition and in field evidenced a good protection level of *Trichoderma* isolates towards soil-borne pathogens, when applied to sugar beet seeds. Such isolates were previously selected for *in vitro* antagonism towards *Pythium* sp., *Rhizoctonia solani* and *Sclerotium rolfsii*, on Potato Dextrose Agar amended with *Trichoderma* culture filtrates. This study aimed at pointing out if further mechanisms could be involved such as rhizosphere competence or induction of resistance. *Trichoderma* isolates were assayed for ability to colonise sugar beet roots, after seed applications as homogenate of liquid culture. A visual rating was utilised to judge the colonisation level of roots plated on *Trichoderma* selective medium two months after the treatment. Roots treated by the selected *Trichoderma* strains showed a high degree of colonisation, superior than that of other applied or natural occurring strains. *Trichoderma* isolates were evaluated also for the ability to induce chitinases in sugar beet leaves as these proteins are considered markers for Systemic Acquired Resistance. Chitinases were detected by a sensitive method on chitin agar plate. One of the selected strains showed the highest chitinase activity compared to the other isolates. Thus the effectiveness of this selected *Trichoderma* strain in controlling soil-borne pathogens under greenhouse and field conditions could be linked to the combined effect of different mechanisms of action, such as antagonism, rhizosphere competence and induction of resistance.

**Key words:** phytopathogenic fungi, plant disease, PR 3

**Introduction**

The potential of *Trichoderma* species as biocontrol agents (BCAs) of plant diseases was firstly recognized in the early 1930's, and in the subsequent years the control of many diseases has been added to the list. One of the most interesting aspects of biological control is the study of the mechanisms employed to exert disease control. The effectiveness of *Trichoderma* as BCA is reported to be based on several mechanisms, such as antibiosis, mycoparasitism, growth promotion and induction of Systemic Acquired Resistance (SAR) (Howell, 2003). The activation of SAR correlates with the expression of Pathogenesis Related (PR) genes, including chitinases, which supposedly act against the pathogen cell walls (Viterbo, 2002). Chitinases are commonly induced upon the attack of pathogens and by various sources of stress. They are therefore classified as PR proteins, which are mainly activated in a salicylic acid dependant manner leading to the expression of SAR (Ziadi et al., 2001).

Previous studies carried out both under controlled conditions and in field evidenced a good protection level of *Trichoderma* isolates towards soil-borne pathogens, when applied to sugar beet seeds (Galletti et al., 2006 a). Such isolates were previously selected for *in vitro*
antagonism towards *Pythium sp.*, *Rhizoctonia solani* and *Sclerotium rolfsii*, on Potato Dextrose Agar amended with *Trichoderma* culture filtrates. This study aimed at pointing out if further mechanisms besides antagonism could be involved in the control of soil-borne pathogens such as rhizosphere competence or induction of resistance.

**Materials and methods**

**In vitro Trichoderma screening for antagonism towards soil-borne pathogens**

Six *Trichoderma* isolates belonging to different species and origin were tested *in vitro* for antagonism towards *Pythium sp.*, *R. solani* and *S. rolfsii* on Potato Dextrose Agar (PDA) amended with *Trichoderma* culture filtrate. The filtrates were prepared in flasks containing 50 ml Potato Dextrose Broth (PDB) with two agar plugs from actively growing PDA colonies of *Trichoderma*, incubated at 28°C for one week in the dark. The liquid cultures were centrifuged at 3000 rpm for 20’, after filtering through sterile cheesecloth. The supernatants, treated twice at 60°C for 30’ to inactivate eventually remaining spores, were added to PDA at 25% (v/v), poured in 9 cm Petri dishes, then one agar plug of an actively growing colony of pathogen was put in the centre and incubated for 4-5 days at 28°C, when maximum diameter of pathogen colonies was recorded compared to control on PDA (three replicates).

**Sugar beet root colonisation**

Sugar beet seeds (cv Aaron) were previously rinsed under tap water for 4 h; six *Trichoderma* isolates with different antagonism ability towards soil-borne pathogens were applied as seven-day old liquid culture homogenate (1×10⁷ CFU ml⁻¹, 1.5 ml gr⁻¹ of seed for 2h); seeds treated with PDB served as control. The seeds were sown in 12 cm diameter pots containing unsterilised peat under greenhouse conditions. After two months, the plants were picked up, the roots were washed, plated on *Trichoderma* selective medium (Elad et al., 1981) and incubated for ten days. The root colonisation rate by *Trichoderma* was then scored (0= no colonisation; 9= complete colonisation).

**Chitinase induction in sugar beet leaves**

*Trichoderma* culture filtrates were prepared as above and water diluted 1:2.5 (v/v). Two leaves per plant at six leaf stage (cv Aaron) were treated twice with 3 ml of the solution at one week interval under greenhouse conditions. Water treated plants were used as controls. Nine plants per each treatment were used. Two and 7 days after the treatments, the total proteins were extracted from samples of untreated leaves and quantified by Bradford assay (Bio-rad). Chitinase activity was detected on chitin agar plate as described by Gohel et al. (2005) with some modifications. Chitin agar plates were prepared using 0.01% soluble glycol chitin (Trudel & Asselin, 1989) in 1% agarose gel. Ten μg of each leaf extract were loaded into wells in the gel and incubated at 37°C overnight. Litic zones around the wells were observed under UV light after staining with 0.01% calcoflour white M2R in 0.5 M Tris HCl pH 8.9. Halo areas were measured by Quantity One software (Bio-rad).

**Results and discussion**

Ba9 (*T. hamatum*) showed the highest growth inhibition towards all the pathogens. B41 (*T. harzianum*) showed strong activity against *Pythium* and *S. rolfsii* but not against *R. solani*, while S2 (*T. virens*) was more active towards *R. solani* and *S. rolfsii* (Table 1). Most isolates showed a superior ability to colonise sugar beet roots than endogenous strains, as showed by the lower value of the control (PDB) (Table 1). In particular S2 and B41 could completely colonise sugar beet roots, while Ba9 showed scarce competence (Table 1).
Table 1. Antagonism ability towards soil-borne pathogens and sugar beet root colonisation by different *Trichoderma* isolates

<table>
<thead>
<tr>
<th><em>Trichoderma</em></th>
<th>Root colonisation (0=no; 9=complete)</th>
<th>Colony growth inhibition (% of control)</th>
<th><em>Pythium</em> spp.</th>
<th><em>R. solani</em></th>
<th><em>S. rolfsii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>B41 (<em>T. harzianum</em>)</td>
<td></td>
<td>9.0</td>
<td>94.2 a*</td>
<td>33.2 b</td>
<td>89.0 a</td>
</tr>
<tr>
<td>S2 (<em>T. virens</em>)</td>
<td></td>
<td>9.0</td>
<td>55.0 b</td>
<td>64.0 a</td>
<td>80.7 a</td>
</tr>
<tr>
<td>Ces</td>
<td></td>
<td>4.5</td>
<td>24.5 c</td>
<td>0.0 e</td>
<td>0.0 c</td>
</tr>
<tr>
<td>Ba9 (<em>T. hamatum</em>)</td>
<td></td>
<td>3.5</td>
<td>95.5 a</td>
<td>64.5 a</td>
<td>87.5 a</td>
</tr>
<tr>
<td>Ba12 (<em>T. longibrachiatum</em>)</td>
<td></td>
<td>3.0</td>
<td>10.0 d</td>
<td>30.0 c</td>
<td>29.5 b</td>
</tr>
<tr>
<td>Lat</td>
<td></td>
<td>5.0</td>
<td>20.1 c</td>
<td>18.2 d</td>
<td>10.0 c</td>
</tr>
<tr>
<td>PDB</td>
<td></td>
<td>3.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Values in columns followed by the same letter are not different for $P\leq0.05$ (LSD test).

Regarding chitinase (PR 3) induction in sugar beet leaves, two days after the first treatment the chitinase activity induced by all isolates was higher than that of the water treated plants, the highest level of PR 3 being observed for S2 isolate (Fig. 1). Moreover, S2 maintained the highest level of PR 3 induction in the time course, while the systemic plant responses to the Ba9 applications seem to be activated only seven days after each treatment (Fig. 1).

In conclusion among the *Trichoderma* isolates only S2 was able to combine all the three mechanisms of action studied. These results suggest the existence of a multiple effect based on antagonism, root colonisation ability and resistance induction accounting for the good diseases control exerted by this strain in repeated trials under greenhouse and field conditions (Galletti et al., 2006 a,b).
References


**Aureobasidium pullulans** strains degrade ochratoxin A in vitro and protect wine grape from ochratoxigenic *Aspergillus carbonarius*

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**Abstract:** Ochratoxin A (OTA) is a possibly carcinogenic mycotoxin produced by *Aspergillus* and *Penicillium* spp. It is a contaminant of different foodstuffs and beverages such as wine. Wine contamination is mainly due to the attack by *Aspergillus carbonarius* on wine-grape. In this study, we assessed the ability of four *Aureobasidium pullulans* strains (LS30, AU14-3-1, AU34-2, AU18-3B) to degrade OTA in vitro and, in lab-scale experiments, their activity as biocontrol agents (BCAs) against *A. carbonarius* on wine grape and their influence on the level of OTA accumulation in berries. All the four strains determined significant decreases of OTA recovery from their growth medium. The less toxic Ochratoxin α (OTα) was the major degradation product. It putatively derived from carboxypeptidase activity of the *A. pullulans* strains. In biocontrol activity assays, the four strains significantly lowered the levels of infections by *A. carbonarius*. Analyses of wine grape treated with the BCAs and inoculated with *A. carbonarius* showed significant decreases of berries contamination with OTA, as compared to untreated control. OTA contamination in samples treated with strains AU14-3-1 and AU18-3B was comparable to Switch™-treated berries, although the fungicide treatment showed no visible symptoms of fungal infection. This suggests a possible active role of AU14-3-1 and AU18-3B in lowering OTA contamination in wine grape berries. Our results encourage further assessments of microbial biocontrol for reducing both fungicide applications and mycotoxin contamination.

**Key words:** biocontrol agents, mycotoxins

**Introduction**

Ochratoxin A (OTA) is a possibly carcinogenic mycotoxin, classified into Group 2B by the International Agency for Research on Cancer, produced by fungi mainly belonging to *Aspergillus* and *Penicillium* spp. OTA contains an isocoumarin fraction (ochratoxin α, OTα), much less toxic than OTA, linked to L-β-phenylalanine by a carboxypeptidase bond.

OTA occurs on a wide range of food commodities and, since 1996, has also been detected in grape juice and red wine (Zimmerli & Dick, 1996). The major responsible for this contamination appears to be *A. carbonarius* (Battilani & Pietri, 2002), following its attack on wine-grape. Wine is considered as the second major source of OTA intake in Europe. Maximum tolerable levels for this mycotoxin both in this beverage and grape juice and wine have been established by EU [(EC) No 123/2005]] as 2 µg L⁻¹.

The most effective methods to lower mycotoxin contamination rely on preventing the attack of mycotoxigenic fungi. Post-production decontamination and/or detoxification methods are also being proposed (Castoria & Logrieco, in press) and microbial degradation of OTA has been reported (Abrunhosa et al., 2002). The integration of preventive measures and detoxification of OTA to less toxic compounds could be a promising strategy to lower wine...
contamination. In this context, microbial biocontrol agents (BCAs) are worth to be investigated, since they act at a preventive level and, if effective against mycotoxigenic fungi, they could display a potential for mycotoxin degradation. Recently, Castoria et al. (2005) demonstrated that the BCA R. glutinis strain LS11 metabolizes the mycotoxin patulin in vitro and on stored apples. It reduces infections of the mycotoxigenic fungus P. expansum and lowers the accumulation of patulin in infected fruits. On the basis of these results, we assessed the ability of four strains of the yeast-like fungus Aureobasidium pullulans to degrade OTA in vitro and, in lab-scale experiments, their biocontrol activity against A. carbonarius on wine grape berries and their influence on OTA contamination.

Material and methods

Four A. pullulans strains were used in all the experiments: LS30, AU14-3-1, AU18-3B, AU34-2. LS30, in particular, had previously been selected for its activity against different pathogens on different crops and characterized for its modes of action (Castoria et al., 2001, 2003) and at a genomic level (De Curtis et al., 2004).

Biological detoxification of OTA by Aureobasidium pullulans strains

The 4 A. pullulans strains were incubated in Lilly Barnett medium with OTA (1 µg mL⁻¹) for 6 days at 23°C on a rotary shaker. Quantitative analyses of OTA and OTα in the medium were performed every 24 h by HPLC/FLD. Experiments were performed twice.

Biocontrol activity of Aureobasidium pullulans strains on grape berries

Mature wine grape berries (cv Montepulciano) were wounded and treated by dipping, alternatively, in Cyprodinil plus Fludioxonil (Switch™) (0.8 mg mL⁻¹) and suspensions of the BCAs (10⁸ cell mL⁻¹). Berries were then sprayed with a suspension of A. carbonarius A1102 conidia (5×10⁴ CFU mL⁻¹, kindly provided by Prof. P. Battilani) and incubated at 24±1°C and 100% R.H. for 6 days, when percentage of infected wounds was recorded. Samples consisted of 5 replicates of 10 berries each. Experiments were performed twice.

OTA and OTα determinations in grape berries

Grape berries were homogenized and diluted with PEG 1% - NaHCO₃ 5%. The extracts were loaded on preconditioned C₁₈ cartridges (Sep-Pak RC), subsequently washed with H₃PO₄ 0.1 M and H₂O. OTA and OTα were eluted with ethyl acetate/methanol/acetic acid (95:5:0.5) and acetonitrile/acetic acid (98:2), respectively. Aliquots of the purified extracts were injected into the chromatographic apparatus.

Analyses were carried out with an Agilent 1100 series LC System. All samples were analyzed with Waters Xterra and Simmetry C₁₈ columns (150×4.6 mm – 5 µm) and a mobile phase of CH₃CN/H₂O/CH₃OOH (99:99:2, v/v/v) at a flow rate of 1.0 ml min⁻¹. OTA and OTα were detected by monitoring fluorescence at 460 nm (λₑₘ; λₑₓ=333 nm). Quantification of OTA and OTα were achieved by comparing peak areas at OTA and OTα retention times with respective calibration curves. OTα standard was obtained by enzymatic hydrolysis of OTA.

Results and discussion

Time-course HPLC analyses of in vitro experiments showed that the main product of OTA degradation was OTα, likely derived from carboxy-peptidase activity of the BCAs. For all the tested strains, decreases of OTA concentration started on day 4 and paralleled increases of OTα, although a statistically significant OTA reduction was recorded only in the case of AU14-3-1 (Fig. 1b). On day 6, decreases of OTA concentration reached 91, 89, 83 and 77% for strains AU34-2, AU14-3-1, AU18-3B and LS30, respectively (Fig. 1c). No significant
adsorption of OTA to the cells of the 4 strains occurred (data not shown). In biocontrol activity assays, strains LS30, AU18-3B and AU14-3-1 displayed significant antagonistic activity, significantly lowering the levels of infections by *A. carbonarius*, as compared to the untreated control (Fig. 2).

![Graph](image_url)

Figure 1. Time-course of *in vitro* OTA decrease and OTα formation analyzed at 0 (upper left), 4 (upper right) and 6 (left) days of incubation with *Aureobasidium pullulans* strains AU34-2, AU14-3-1, AU18-3B and LS30. Values with the same letters are not significantly different at P=0.01 (*P*=0.05 for Fig. 1b) according to Duncan’s multiple range test. Letters in boxes above columns refer to Duncan’s test for OTα determinations.
Figure 2. Infection percentages on wine grape berries inoculated with *Aspergillus carbonarius* strain A1102 and treated with *Aureobasidium pullulans* strains LS30, AU18-3B, AU14-3-1, AU34-2 or the fungicide Switch™. Percentage values were converted into Bliss angular values before statistical analysis. Values with the same letters are not significantly different at $P=0.01$ according to Duncan’s multiple range test.

Figure 3. HPLC analyses of extracted berries inoculated with *Aspergillus carbonarius* strain A1102 and treated, alternatively, with the *Aureobasidium pullulans* strains LS30, AU18-3B, AU14-3-1, AU34-2, or the fungicide Switch™. Values with the same letters are not significantly different at $P=0.01$ according to Duncan’s multiple range test. Letters in boxes above columns refer to Duncan’s test of OTA determinations.

HPLC analyses of berries pretreated with the *A. pullulans* strains showed striking decreases of OTA contamination, as compared to the control (Fig. 3). The pattern of contamination mirrored the activity of the BCAs: the higher the antagonistic activity the lower the OTA contamination. Therefore, the observed OTA decreases could be due to a
delay in the development of *A. carbonarius* in BCA-pretreated but infected berries. Additional mechanisms such as interference in OTA biosynthesis or toxin degradation by the BCAs cannot be ruled out yet. In particular, OTA contamination in samples treated with AU14-3-1 and AU18-3B was comparable to Switch™-treated berries, although the fungicide yielded no visible symptoms of infection. This datum suggests a possible active role of these strains in lowering OTA contamination in wine grape berries. As regards degradation, low amounts of OTα were detected in all treatments, although the ratio OTα/OTA was higher in BCA-treated berries. Since ochratoxigenic fungi can degrade OTA (Bejaoui et al., 2006), in our experimental conditions it was not possible to ascertain whether part of detected OTα is due to enzyme activity of *A. pullulans* strains. Whatever the mechanism(s) involved in OTA reduction by these strains, our results encourage further assessments of microbial biocontrol as a valuable strategy for reducing fungicide applications as well as mycotoxin contamination.

References


Protection of grapevine against gray mold disease and activation of chitinase and β-1,3-glucanase by native rhizobacteria under field conditions

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Abstract: In a recent study, we have characterized some non-pathogenic bacteria originally isolated from vineyards that are capable of eliciting resistance reactions in grapevine toward *Botrytis cinerea* using in vitro system (Trotel-Aziz et al., 2006). Based on these findings, we investigated the effectiveness of these bacteria in inducing resistance of grapevine against gray mold disease under field conditions. Fungal disease and defense reactions (chitinase and β-1,3-glucanase activities) of grapevine plants treated with bacteria were followed in leaves and berries over three years (2003 - 2005) in Champagne area, France. We compared three methods of bacterial inoculation: syringe infiltration into buds, foliar spray and soil drenching. We have shown that selected bacteria reduced disease and enhanced chitinase and β-1,3 glucanase activities in leaves and berries. The importance of these responses was dependent on the bacterial strain and method of inoculation. Moreover, soil drenching provided the most consistent protection of leaves and berries against *B. cinerea*. With this method, bacterial strains and *B. cinerea* are considered spatially separated, indicating that native bacteria conferred disease resistance mainly through the induction of plant defense reactions.

Key words: induced systemic resistance, non-pathogenic bacteria, *Vitis vinifera*

Introduction

Grapevine is affected by several fungal diseases, among them gray mold caused by *Botrytis cinerea* inflicts serious damage and yield loss in French vineyards. This disease is usually controlled by chemical fungicides, but the environmental impacts and the appearance of resistant strains call for new technologies, such as using biocontrol agents or natural compounds to induce the resistance of the host plant (Aziz et al., 2003, Barka et al., 2002, Trotel-Aziz et al., 2006).

Non-pathogenic bacteria have been shown to provide plant protection against several pathogens either directly by competition and antibiosis or indirectly by inducing systemic resistance (ISR) of the host plant (van Loon et al., 1998). The mechanisms involved in ISR appear to vary among bacterial strains and pathosystems. In some cases, ISR is characterized by a systemic accumulation of PR proteins (Bargabus et al., 2002), while in other cases it is associated with different mechanisms (Verhagen et al., 2004).

Recently, we have isolated seven non-pathogenic bacteria from vineyards, identified as *Acinetobacter lwofii* (PTA-113 and PTA-152), *Bacillus subtilis* (PTA-271), *Pantoea agglomerans* (PTA-AF1 and PTA-AF2) and *Pseudomonas fluorescens* (PTA-268 and PTA-CT2), that are capable of eliciting resistance of in vitro-grown grapevine toward *B. cinerea* (Trotel-Aziz et al., 2006). In this study, we sought to evaluate the effectiveness of these
bacteria in inducing resistance reactions in grapevine plants against *B. cinerea* under field conditions.

**Material and methods**

*Field experiments*
Experiments were conducted from 2003 to 2005 in two different vineyards (CR and NL) with a susceptible grape cv. Chardonnay in the Champagne area, France. Bacteria were grown in LB medium before they used in trials at 10^8 cfu/ml. They were applied by infiltration, foliar spray and soil drenching in the first vineyard, and only by soil drenching in the second.

*Botrytis cinerea challenge and disease evaluation*
Grapevine leaves were excised from the top of shoots 2 months after application of bacteria and challenged with a conidial suspension of *B. cinerea* as described by Aziz et al. (2003). Disease was measured as the average diameter of necrotic lesions formed 7 days post-challenge. In berries, disease severity was estimated at harvest under natural contamination.

*Chitinase and β-1,3-glucanase activities*
Grapevine leaves and berries without visible symptoms were harvested during growing seasons and stored at –80°C. Chitinase and β-1,3-glucanase activities were assayed according to the method of Wirth & Wolf (1992) with carboxymethyl-chitin-remazol brilliant violet and carboxymethyl curdlan-remazol brilliant blue as respective substrates.

**Results**

*Protection of grapevine leaves against *B. cinerea* depends on the bacterial strain and method of inoculation*
Gray mold symptoms were generally reduced in the bacterized plants, with some dependency on strain and inoculation method. With the bacterial infiltration, the leaf disease was reduced in the presence of PTA-152, PTA-AF1, PTA-268 by 20 to 50% compared to the control (Fig. 1A). Spray application also results in a significant disease reduction with PTA-AF1 and PTA-CT2 (39-43%) (Fig. 1B). When applied by soil drenching, PTA-113, PTA-AF1, PTA-AF2 and PTA-CT2 provided a significant reduction of disease from 28 to 49% (Fig. 1C). For the majority of bacteria, soil drenching provided the greatest biocontrol efficiency.

*An assessment of induced systemic resistance in grape plants by native bacteria*
To assess ISR, selected bacteria were applied by soil drenching and protection was assayed in leaves and berries. In control leaves, gray mold symptoms were characterized by large necrotic lesions, whereas in bacterized plants they were much less severe (Fig. 2A). PTA-113, 152, 268 and CT2 induced a significant reduction of disease (45-70%) (Fig. 2A). AF1 also led to a significant protection, while AF2 and 271 remained less efficient. In berries a mean of 55% of disease severity was recorded in control plants under natural contamination (Fig. 2B). Clusters from PTA-AF1 and AF2-treated plants exhibited a protection of about 26 - 36%. 271 also resulted in a slight protection of berries, while the other strains were ineffective (Fig. 2B).

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55% of disease severity was recorded in control plants under natural contamination (Fig. 2B). Clusters from PTA-AF1 and AF2-treated plants exhibited a protection of about 26-36%. Also resulted in a slight protection of berries, while the other strains were ineffective (Fig. 2B).

![Figure 1](image1.png)

**Figure 1.** Reduction of gray mold disease symptoms in grapevine leaves by native bacteria as related to the method of bacterial inoculation. Bacteria were applied at the beginning of 2003 and 2004 growing seasons, by infiltration (A) at bud burst (10 ml/plant), spray (B) on leaves (100 ml/plant) or soil (C) drenching (200 ml/plant). * indicates that values are significantly different (Duncan’s test P≤0.05).

![Figure 2](image2.png)

**Figure 2.** Reduction of gray mold disease in grapevine leaves (A) and berries (B) by native bacteria. Bacteria were applied by soil drenching at 10^5 cfu/ml (150 ml/plant) at the beginning of 2004 season. * indicates that values are significantly different (Duncan’s test P≤0.05).

**Chitinase and ß-1,3-glucanase activities in grapevine leaves**

To provide insights into defense reactions associated with the induction of resistance in grapevine, chitinase and ß-1,3-glucanase activities were monitored in both leaves and berries of control and bacteria-treated plants by soil drenching during 2004 season.

In leaves (Fig. 3), a significant increase of chitinase activity was observed at flowering (Fig. 3A,B), reaching maximum values in bacterized plants within 2 months after soil drenching (22 July). Highest chitinase activity was achieved with PTA-CT2 followed by 268 and 113. ß-1,3-glucanase activity increased in leaves of all bacterized plants (Fig. C,D), with a maximum activity after version (Sept.). ß-1,3-glucanase was highest and equivalent in response to PTA-113, 152, 271, AF1 and CT2.
Chitinase and $\beta$-1,3-glucanase activities in grapevine berries

Chitinase and $\beta$-1,3-glucanase activities showed inverse patterns in berries during the growing season (Fig. 4). Chitinase activity increased gradually in both control and bacterized plants starting from véraison until harvest. In berries of bacterized plants (except those treated with PTA-AF2), chitinase level was 2 to 5-fold above that observed in the control plants (Fig. 4A, B). $\beta$-1,3-glucanase activity was detected in berries of control plants before véraison and decreased progressively until harvest (Fig. 4D). The most $\beta$-1,3-glucanase activity was achieved with PTA-AF1 and CT2, followed by PTA-271.

![Graphs showing chitinase and $\beta$-1,3-glucanase activities](image)

Figure 3. Induction of chitinase (A,B) and $\beta$-1,3-glucanase (C,D) activities in grapevine leaves by native bacteria. Individual bacteria were applied by soil drenching at $10^8$ cfu/ml at the beginning of 2004 season. * indicates that values are significantly different (Duncan’s test $P \leq 0.05$).

![Graphs showing chitinase and $\beta$-1,3-glucanase activities](image)

Figure 4. Induction of chitinase (A,B) and $\beta$-1,3-glucanase (C,D) activities in grapevine berries by native bacteria. Individual bacteria were applied by soil drenching at $10^8$ cfu/ml at the beginning of 2004 season. * indicates that values are significantly different (Duncan’s test $P \leq 0.05$).

Discussion

Experiments conducted in vineyards show that selected bacteria clearly confer distinct protection in grapevine against $B.\ cinerea$. The level of this protection depends on the bacterial strain and on the method of bacterial inoculation. Highest biocontrol efficacy was achieved with $P.\ agglomerans$ (PTA-AF1 and AF2) and $P.\ fluorescens$ (268 and CT2) when compared to $A.\ lwoffii$ (113 and 152) and $B.\ subtilis$ (271). Moreover, of the three examined methods of bacterial inoculation, soil drenching provides the most consistent protection of leaves and berries against $B.\ cinerea$. With this method, bacterial strains and $B.\ cinerea$ are
considered spatially separated, indicating that native bacteria conferred protection against the pathogen mainly through the induction of plant resistance. Accordingly, the bacteria-induced protection is associated with a stimulation of systemic activities of chitinase and β-1,3-glucanase in grapevine plants.

Acknowledgements

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References


Mechanism of action of *Streptomyces rochei* in combination with *Trichoderma harzianum* for the biocontrol of *Phytophthora* root rot of pepper

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**Abstract:** We analyse the action mechanism of the bacteria *Streptomyces rochei* “Ziyani” isolated by our group from the rhizosphere of pepper plants for its potential use in combination with the fungus *Trichoderma harzianum*. Both antagonists can be used for the biological control of root rot caused by the pathogenic oomycete *Phytophthora capsici* in pepper plants. When the antagonistic capacity of the bacteria *Str. r.* “Ziyani” was analysed in *in vitro* confrontations with the pathogen *P. capsici*, an inhibition zone was obtained, demonstrating the production by the bacteria of compounds with antifungal activity. To identify these compounds, a bioassay using thin layer chromatography was carried out in silica gel of the discharges of *Str. r.* “Ziyani to the liquid culture medium of potato-dextrose. The compound was purified by HPLC and identified by mass spectrometry as 1-propanone, 1-(chlorophenyl). The production of this antibiotic of great anti-oomycete capacity, which is responsible for the antagonism of the bacteria, may well be one of the main mechanisms for inhibiting pathogen growth; along side the mechanisms used by the antagonist fungus *T. harzianum*. The use of both antagonists added together in formulated vermiculite, culture medium and plantation earth significantly reduced the incidence of root rot in pepper plants by 74.8% compared with the control.

**Key words:** Biocontrol, pepper, *Phytophthora capsici*, root rot, *Streptomyces rochei*

**Introduction**

Root rot of peppers (*Capsicum annuum*) caused by the oomycete *P. capsici* is responsible for great losses in crops world-wide. The disease is fatal and, by the time that the first symptoms become evident, the tissues are already totally invaded and the plant dies within a few days (Sid Ahmed et al., 1999). The way to improve plant protection may be to use combinations of biocontrol agents, especially when they exhibit complementary modes of action, a supposition which has been proved correct using different bacterium/ffungus combinations (Elad et al., 1994; Guetsy et al., 2002; Li et al., 2005). In an attempt to improve the effectiveness of biocontrol, we have studied the combination of agents possessing different action mechanisms, in this case, the fungus *T. harzianum* which exhibited antagonistic activity against *P. capsici* and a bacterial isolate, *Str. r.* “Ziyani” which was obtained from the rhizosphere of pepper plants (Ezziyyani, 2004).
Material and methods

**Plant materials, pathogen and biocontrol agents**

*Capsicum annuum* L., cv. California Wonder, disinfected seeds were grown in 4×4×14-cm pots containing a mix of autoclaved peat, sand and vermiculite (3:1:1/2) placed in a growth chamber and maintained at 21±3°C, 78% relative humidity and 12-hr photoperiod. After 3 months, seedlings were transferred to greater pots or soil where they were treated. The causal agent of pepper root rot, *P. capsici* isolate 15, was used from our culture collection (Candela et al., 1995). *T. harzianum* was the fungal biocontrol agent used. The isolate 2413 came from the Colección Española de Cultivos Tipo (CECT) from Valencia, Spain. *Str. r.* “Ziyani” was used as the bacteria biocontrol agent. This was isolated by us from the rhizosphere of a healthy pepper grown and was selected for its *in vitro* anti-oomycete activity against *P. capsici* and for its symbiosis with *T. harzianum*. The bacteria were maintained in NA (Nutritive Agar, Merck) medium. The *P. capsici* inoculum was prepared in “Vermiculite-PDB” medium (Ezziyyani, 2004). The *T. harzianum* inoculum was prepared in the vermiculite-Avy3 medium (water-oat at 15%) in the same way as for *P. capsici*.

**Isolation and detection of the anti-oomycete substance produced by the bacteria* Str. rochei “Ziyani”*

Five discs were taken from 7-day cultures of *Str. r.* “Ziyani” in NA medium and placed in 1 litre of NB medium. The culture broth was incubated at 28°C on a rotary shaker at 130 rpm for 20 days and then centrifuged at 1,300 g for 30 min. The supernatant was filtrated through a Whatman nº 2 filter paper and was extracted with n-buthanol: ethyl-acetate (50:50, v/v) added to the extract in the proportion 2:1. The organic phase was evaporated to dryness, suspended in methanol and, kept at 4°C until use.

The extraction products were separated by thin-layer chromatography (TLC) on a silica gel 60 plate (Merck) for 35 min with Chloroform-Methanol (70:30) elution solvent. The activity against *P. capsici* was examined on TLC impregnated with PDA and conidia suspension of *P. capsici*. After three days the Rf of the oomycete mycelium growth inhibition zone was calculated. In the rest of the chromatoplate the inhibition zone was eluted with ethanol, filtered and concentrated with N2 and kept at 4°C until use. The compounds were purified with C18 reverse-phase high-performance liquid chromatograph (HPLC) equipped with G1315 diode-array detector. The analytical column was a LiChroCART 250×4 mm I.D. packed with LiChrospher 100, RP-18 e (5-µm spherical particles). The anti-oomycete substances were eluted using a linear gradient solvent system from 10% acetonitrile in water Milli-Q to 100% acetonitrile at a flow-rate of 1.0 ml/min. The elution protocol lasted 10 min. at room temperature. Each eluate was concentrated with N2, re-dissolved in methanol and evaluated against *P. capsici*. The compound showing anti-oomycete activity was identified by mass spectrometry in a chromatograph Agilent, 5973 (G2577A detector).

**Results**

The inhibition zone arising from the action of the antagonist *Str. r.* “Ziyani” led us to try to identify the anti-oomycete substance produced by the bacteria. The extract obtained and separated by TLC, provided a visible band at 360 nm, 12 mm wide and with an Rf of 0.8. After purification by HPLC, the major band was registered at 248 nm, and two main peaks were obtained, the first with a retention time (RT) of 2.795 and 55.8% intensity, and the second with an RT of 3.819 and 34.4% intensity. The anti-oomycete test carried out in a Petri dish confirmed that only the compound with an RT=2.795 showed antagonistic activity in the face of *P. capsici*. Identification based on spectral data and NMR showed it to be
1-propanone, 1 (4-chlorophenyl). The anti-oomycete activity was tested at 24, 48, 72 and 96 hours. The growth kinetics of the pathogen showed an increasingly reduced diameter in its confrontation with the substance. Controls were carried out with water and with ethanol, in which the purified compound was dissolved during HPLC. The anti-oomycete compound was active from 24 hours onward, slowing the growth of the pathogen and inhibiting expansion of the P. capsici colony above a concentration of 10 µg/ml.

**Evaluation of different treatments on root rot caused by P. capsici in vivo**

The treatment was continued for two months with the following results: all the infected non-treated plants (I+NT) died, while all the treated non-infected plants (T+NI) survived, meaning that the pathogen was sufficiently virulent to kill the plants if they were not treated with the biocontrol and that the biocontrol agents are innocuous for the plants. As regards the infected and treated plants (I+T), the dose of antagonistic bacteria affected the time at which the root rot symptoms appeared: the lowest doses of 1.9×10⁴ CFU /ml led to the highest number of plants dying, while the highest doses delayed the appearance of symptoms (and hence the disease), some plants even flowering before they died. The greatest reduction in mortality was obtained with the isolate **Str. r. “Ziyani”** at a concentration of 1×10⁹ CFU/ml. The use of different concentrations of the fungus **T. harzianum**, on the other hand, did not influence the appearance of the disease.

In soil assays, the individual and combined addition of the antagonists were tested, using 35.0×10⁷ conidia/ml of **T. harzianum** and 1.0×10⁹ CFU /ml of **Str. r. “Ziyani”**. The percentage of root rot reduction was the same in the fifty plants that were treated. The most effective was 74.8% reduction in root rot when the infected plants were treated with the combination of both antagonists.

**Discussion**

In this work, we identify an antibiotic produced by the isolate **Str. r. “Ziyani”** as being partly responsible for the in vitro inhibition of pathogen growth and, therefore, as playing an important part in the biocontrol mechanism that reduce root rot caused by P. capsici in peppers in vivo. To date, several **Streptomyces** antibiotics have been isolated which are known to be active against fungal pathogens (Kim et al., 1999) and our results agree with these authors. The first conclusion that can be reached from the results using pots is that both antagonists can be added together since they are symbiotic and their joint effect is greater. In the assays, good results were obtained, especially regarding the high proportion of conidia and propagules that directly influence the life of the bio-preparation in the field and its effect as biocontrol agent. The addition of plantation soil particles both to the vermiculite and liquid culture media encouraged the adaptation of the antagonists. It also prevented the particles adhered to the vermiculite from being washed to the bottom of the flasks when the culture media were added. One of the reasons for our success was to have encouraged the compatibility of two antagonists from different genera, as demonstrated in in vitro confrontations between the two antagonists that inhibit pathogen growth, which use a variety of mechanisms, including antibiosis, mycoparasitism, enzymatic lysis and a notable competition for space and nutrients. The in vivo assay enabled us to confirm the innocuous nature of the antagonists, while the use of the antagonists in combination rather than individually was effective at reducing the root rot caused by P. capsici by up to 75%. Lastly, the results serve as a justification for using a combination of compatible antagonists selected for their different biological capacities and confirm the suggestion of other authors (Bora et al., 2004; Akköprü & Demir, 2005), that the use of more than one biocontrol agent is necessary for reducing the vagaries of biological control. By using the characteristics of a
combination of antagonists, we open up the possibility of designing future strategies for the biological control of *P. capsici* in the soil and, possibly, of transferring the knowledge gained to the control of other pathogens.

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**References**


Genetic strategies for the selection of enhanced rhizosphere colonization in biocontrol bacteria antagonistic towards *Rosellinia necatrix*

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**Abstract:** Biological control of fungal diseases is usually based on the application of microorganisms selected for their capacity to produce *in vitro* antifungal factors. However, recent studies on bacterial behaviour in natural environments have shown the importance of the competence with other microorganisms for efficient colonization of a specific ecological niche, becoming root-colonization one of the limiting step in biological control. In this sense, plants can be inoculated with so called biocontrol bacterial but their success will depend on the establishment on and along the growing root system. In a previous study, forty-one bacterial strains were isolated from the roots of symptomless avocado trees; ten of them could prevent *in vitro* growth of *Rosellinia necatrix*, the causal agent of avocado white root rot. The ability of several of these antagonistic strains to colonize the roots of avocado was tested *in vivo* and, two of them, were selected by their colonization efficiency: *Pseudomonas alcaligenes* strain GBF.1.11 and *P. pseudoalcaligenes* GBF.2.18. None of these strains produced detectable antibiotics, a characteristic which is desirable for commercialization purposes. Biocontrol assays against *R. necatrix* showed that while GBF.2.18 could prevent fungal growth, GBF.1.11 did not. Two different genetic strategies were used to enhance root tip colonization on these two isolates: a) direct selection from a mini-Tn⁵ pool of mutants and, b) knock-out of the *mutY* gene; loss of this gene function has been reported to increase the frequency of spontaneous chromosomal mutations in a *P. fluorescens* strain.

**Key words:** enhanced colonization, *Pseudomonas*, *mutY* locus

**Introduction**

Certain *Pseudomonas* strains protect plants from diseases caused by soil-borne pathogenic fungi. Besides the ability of such strains to stimulate host defences, and/or to perform direct antagonistic effects on the pathogen, the biocontrol capacity depends essentially on aggressive root colonization of the plant to be protected. Competitive colonization not only plays an important role in biocontrol but also in processes such as rhizoremediation, phytostimulation and biofertilization (de Weert, 2004). Future isolation of strains derivatives showing improved colonization ability require first a better understanding of the traits involved in root colonization. Genetic engineering has been applied to improve the competence of antagonistic strains showing low colonization ability. In this sense, introduction into poor colonizers of the *sss* gene, encoding a site-specific recombinase involved in DNA rearrangement, was shown to improve the ability of the strains to colonize root tips (Dekkers et al., 2000). A mutation into
the mutY gene, encoding a mismatch-correction glycosylase is involved in enhanced colonization ability (de Weert et al., 2004).

In a previous screening for biocontrol bacteria against R. necatrix, causal agent of avocado white root rot, forty-one bacterial strains were isolated from the roots of symptomless avocado trees located in orchards affected by this pathogen. Ten of these strains could prevent fungal growth in vitro. Persistence of some of these isolates in the rhizosphere of avocado plants was tested after artificial inoculation and two of them, P. alcaligenes strain GBF.1.11 and P. pseudoalcaligenes GBF.2.18, were selected for their ability to establish in the rhizosphere of avocado plants at about 10^5-10^6 colony forming units (C.F.U.) per gram of fresh root 50 days after inoculation. In spite of the higher number of antagonistic properties shown in vitro by GBF.1.11, this strain was not able to prevent fungal growth in vivo, however, GBF.2.18 showed biocontrol ability against R. necatrix. The final aim of this work is to isolate derivatives of these two strains showing enhanced rhizosphere colonization. Two different genetic strategies were used for the isolation of these derivatives: mini-Tn5 mutagenesis and knockout of the mutY gene.

Material and methods

Bacterial strains and culture conditions
The bacterial strains used in this study are listed in Table 1. King’s B medium was used for culturing bacterial strains at 24°C. Luria-Bertani medium was used for culturing Escherichia coli strains at 37°C. Bacterial strains were stored for preservation at -80°C in 50% glycerol. Media were supplemented when required with Kanamycin (Km) and/or nitrofurantoin (Nf) at a final concentration of 25 and 100 µg/ml, respectively.

Mini-Tn5 mutagenesis
Mutants of GBF.1.11 and GBF.2.18 were obtained by random transposon mutagenesis using triparental mating. Matings of both strains were performed using as donor an E. coli strain harbouring plasmid pRL1063A, which contains a Tn5luxAB transposon and a KmR gene. An E. coli strain carrying plasmid pRK2013 was used as helper strain. Trans-conjugants were selected in media containing Km and Nf.

Growth of avocado plants
Avocado plantlets were obtained following in vitro germination of avocado embryos as described by Pliego-Alfaro (1988). Seedlings were grown in perlite in a growth chamber for 20-30 days in order to harden the roots, after which the avocado plants were ready to be used in biocontrol experiments.

Construction of knock-out mutations in the mutY gene
Plasmid pMP5572 was introduced into Pseudomonas strains GBF.1.11 and GBF.2.18 by triparental mating using helper plasmid pRK2013. Single homologous recombinants in mutY were selected in KB medium supplemented with Km and Nf.

DNA manipulations
Basic DNA and molecular techniques were performed following standard methods. Genomic DNA was extracted using the Jet Flex Extraction Kit (Genomed; Löhne, Germany). EcoRI and BamHI digestions were carried out following the instructions provided by the manufacturer. DNA hybridization was performed following standard methods (Sambrook et al., 2001), using a DIG- Nucleic Acid Detection Kit (Roche; Mannheim, Germany) and following the instructions provided by the manufacturer. DNA was transferred onto a nylon membrane by upward capillary transfer, and cross-linked by UV irradiation. Prehybridization and hybridization stages were carried out at 65°C. Neomycin phosphotransferase (npt)
resistant gene probe was labelled by PCR reaction with chemiluminescent digoxigenin-dNTPs using a DIG Labelling Mix (Roche; Mannheim, Germany), primers Nm-F (5′-TGAACAAGATGGATTGCACG-3′) and Nm-R (5′-AGAAGAACTCGTCAAGAGG-3′) and DNA template.

Results and discussion

Isolation of enhanced root tip colonizing derivatives of GBF.1.11 and GBF.2.18 by mini-Tn5 mutagenesis

Efficient rhizosphere colonizers can be obtained after inoculation of seedlings with a mixture of different bacteria followed by growth of the seedlings in a gnotobiotic sand system (Simons et al., 1996). Utilization of this method during several cycles with a specific strain leads to enhanced root tip colonizing derivatives of the wild-type strain (Kuiper et al., 2001). This procedure has been previously used to isolate derivatives of *P. fluorescens* WCS365 colonizing efficiently both the dicotyledonous plant tomato as well as the monocotyledonous plant grass (de Weert et al., 2004).

A similar procedure was applied in the rhizosphere of avocado plants to a random collection of mini-Tn5-*luxAB* marked mutants derived from strains GBF.1.11 and GBF.2.18. After growth of each of these two mutant collections in KB medium at 28ºC, the cells were washed, resuspended in PBS buffer and used to inoculate the roots of one-month-old avocado *in vitro* germinated plantlets. Roots were placed in a sterile tube with moistened sterile vermiculite and grown as described above. Bacteria were isolated from the root tip after ten days and grown on KB plates supplemented with Km. Single colonies were scraped together from the plates and used to inoculate fresh KB medium. After overnight growth, the cells were washed in PBS and avocado roots were inoculated as previously described. These cycles of enrichment were carried out three times (Fig. 1).

| Table 1. Bacterial strains used in this study |
|-------------------------------|---------------------------------|
| **Bacterial Strains**         | Relevant characteristics\(^a\) | Reference or source         |
| *E. coli* DH5\(\alpha\)       | Used for transformation and propagation of plasmids | (Hanahan, 1983) |
| *P. alcaligenes* GBF.1.11     | Wild type, Nm\(^r\)             | This study                   |
| *P. pseudoalcaligenes* GBF.2.18 | Wild type, Nf\(^r\)           | This study                   |

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<tr>
<th><strong>Plasmids</strong></th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>pMP5572</td>
<td>Harbouring part of PCR fragment of <em>mutY</em> used for homologous recombination; Km(^r)</td>
<td>(de Weert et al., 2004)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for triparental mating; Nm(^r), Km(^r)</td>
<td>(Ditta et al., 1980)</td>
</tr>
<tr>
<td>pRL1063A</td>
<td>Plasmid harbouring promoterless Tn5<em>luxAB</em>, Km(^r)</td>
<td>(Wolk et al., 1991)</td>
</tr>
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\(^a\)Km – Kanamycin; Nm – Neomycin; Nf – nitrofurantoin; \(^r\) – resistant

After three cycles of enrichment, six and eight different colonies were isolated from the mutant collections of GBF.1.11 and GBF.2.18, respectively. The strains isolated were characterized according to their colony morphology, API test and other characteristics known
to be involved in bacterial root colonization such as: a) motility: swimming, swarming and twitching and, b) the production of N-acyl homoserine lactones (AHLs) involved in quorum sensing regulation. Three out of the six mutants strains derived from GBF.1.11 showed reduced twitching motility (data not shown).

![Diagram](image)

**Figure 1.** Isolation of competitive root tip colonizing bacterial strains on avocado plantlets. Mini-Tn5 mutants were subjected to three cycles of enrichment

Currently, we are analysing the rhizosphere colonization and biocontrol ability of these mutant strains in competition with their corresponding parental strains. Amplification and sequencing of the regions flanking the transposon borders should allow us to identify the exact point of the insertion of the transposon into genome of the strains as well as the gene function affected by the transposon.

**Southern analysis of mini-Tn5 mutants**

Mini-Tn5 derivatives are readily transposable in most bacteria by conjugation via suicide vectors, transduction or transformation. In most tested cases, transposition occurs randomly into the chromosome of the recipient strain causing inactivation of host genes and positional effects affecting gene expression (Sousa et al., 1997).

To determine whether the strains isolated contain the transposon in different chromosomal positions we used Southern blot analysis using a neomycin phosphotransferase (npt) probe, the results obtained are shown in Fig. 2. In all cases, a single hybridization band was observed. As the npt probe does not contains restriction sites for the enzymes used, this result was expected for single insertions of the transposon into the genome of each mutant. Hybridization signals were found on different-sized restriction fragments, suggesting that transposition occurred randomly into the genome of both strain.
Figure 2. (A) Southern blot analysis of genomic DNA isolated from mini-Tn5 mutants derived from strain GBF.2.18. and digested with BamHI (A.1) or EcoRI (A.2). (B), Southern blot analysis of genomic DNA isolated from mini-Tn5 mutants derived from strain GBF.1.11 and digested with EcoRI.

Figure 3. Generation of mutY mutants by single homologous recombination using an E.coli strain containing helper plasmid pRK2013

*Generation of mutY mutants*

A second strategy for the selection of enhanced root tip colonizing strains was to generate mutY mutants. Bacteria with a mutY mutation are unable to repair their A:G mismatches which results in a higher number of mutations per cell (Miller, 1996). de Weert et al. (2004) related these unrepaired mutations, combined with enrichment procedures, with a superior colonizing phenotype of the selected strains due to specific combinations of mutations, present in some mutant cells, which are beneficial for colonization. Single homologous recombinants of mutY genes were obtained for GBF.1.11 and GBF.2.18 as shown in Fig. 3.
Currently, we are carrying out cycles of enrichment in the rhizosphere of avocado plantlets to select combinations of mutations leading to an enhanced colonization of the avocado rhizosphere.

References


Induction of phytoalexin synthesis, chitinase and β-1,3-glucanase in grapevine leaves by chitosan, and resistance to Botrytis cinerea

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Abstract: Chitosan, β-1,4-linked N-glucosamine, is a deacetylated derivative of chitin which is an important constituent of cell walls of many fungi. In this study, we investigated the elicitor activity of crustacean-derived chitosan oligomers with a wide range of known molecular weight (MW) and degree of deacetylation (DDA) in grapevine leaves. We showed that chitosan oligomers are potent elicitors of phytoalexins, trans- and cis-resveratrol, ε-viniferins and piceids. The amount of elicited-phytoalexins was dependent upon Molecular Weight (MW) and Degree of DeAcetylation (DDA) of chitosan. The trans-isomers were more influenced by the chitosan structure than cis-isomers. In all cases, chitosan with a low MW and a DDA ≥80% exhibited high phytoalexin-inducing activity. The most active chitosan also led to marked stimulation of chitinase and β-1,3-glucanase activities in grapevine leaves. This elicitor capacity of chitosan appeared to be associated with an induced protection against gray mold. This protection apparently did not result from direct effect of chitosan on fungal pathogen growth but rather from an induction of plant resistance.

Key words: gray mold, induced resistance

Introduction

Gray mold, caused by the fungus Botrytis cinerea, is among the most damaging diseases of grapevine (Vitis vinifera L.). Control of this pathogen is generally achieved with chemical fungicides, but the appearance of fungicide-resistant strains and negative environmental impacts have intensified the need for alternative disease management methods. In recent years, the importance of oligosaccharides as disease control agents has been emphasized (Agrawal et al., 2002; Trotel-Aziz et al., 2006; Vander et al., 1998). Chitosan, a β-1,4-linked glucosamine, is a totally or partially deacetylated derivative of chitin. It has been shown to elicit a variety of defense reactions in higher plants such as the stimulation of phenylalanine ammonia lyase (PAL), peroxidase and lipoxygenase activities, as well as the accumulation of phytoalexins and PR proteins (Agrawal et al., 2002; Trotel-Aziz et al., 2006). In this study, we investigated the elicitor activity of chitosan (CHN) oligomers with different Molecular Weight (MW) and Degree of DeAcetylation (DDA) through phytoalexin accumulation in grapevine leaves. We further examined the ability of the most active CHN to induce chitinase and β-1,3-glucanase activities, and to protect grapevine leaves against B. cinerea.
Material and methods

**Biological materials and treatments**
Grapevine plantlets (*Vitis vinifera* cv. Chardonnay) were obtained by multiplication in vitro. Leaves were excised from 10-week-old grapevine plantlets and incubated on a reference buffer as described in Aziz et al. (2003). The elicitation process was carried out with CHN fragments differing in their MW and DDA.

**Defense reactions**
Phytoalexins were extracted and analyzed by HPLC as described by Aziz et al. (2006). Chitinase and β-1,3-glucanase activities were determined as described by Wirth & Wolf (1992) using carboxymethyl/chitin/remazol brilliant violet 5R and carboxymethyl-curdlan-Remazol-Brilliant Blue as respective substrates.

**Protection assays**
Detached leaves were pre-incubated on 2 mM MES buffer, pH 5.9, containing various concentrations of CHN. After 48 h, leaves were challenged with a conidial suspension of *B. cinerea* (Aziz et al., 2003), and disease development was measured as the average diameter of lesions formed 6 days post-inoculation.

![Graphs showing phytoalexin accumulation](image)

Figure 1. Induction of phytoalexin accumulation in grapevine leaves by chitosan oligomers with different MW and DDA. Leaves were floated on buffer with or without chitosan at 200 µg/ml, pH 5.9. Control buffer (open square), chitosan of low MW and DDA 80% (solide square), chitosan of low MW and DDA 98% (open triangle), chitosan of high MW and DDA 80% (solide triangle), chitosan of high MW and DDA 70% (open circle).

**Results and discussion**
Resveratrol (*trans*-3,4′,5-trihydroxystilbene) and its dehydrodimer ε-viniferin are considered as the major phytoalexins produced by grapevine plants under biotic and abiotic stress, and are often associated with pathogens resistance (Aziz et al., 2006; Jeandet et al., 2002). Induction of *trans*- and *cis*-resveratrol and derivatives, viniferin and piceid, was observed in grapevine leaves after treatments with chitosan oligomers differing in MW and DDA (Fig. 1).
The production of ε-viniferin and piceid suggests that chitosan can induce a rapid dimerization and glycosylation of resveratrol, respectively. Alternatively, ε-viniferin could also be produced through an oxidative coupling of resveratrol catalyzed by a cell-wall localized peroxidase (Calderon et al., 1994). For all chitosans, the production of phytoalexins showed similar profiles, and the trans-isomers remained predominant to the cis-isomers. Amounts of phytoalexins were generally greatest with chitosan of low MW. The larger oligomers (with high MW) were less active. The DDA appeared also to influence the elicitor activity of chitosan. The maximum production of resveratrol, ε-viniferin and piceid was observed with a DDA of 80%, indicating that both MW and DDA can modulate chitosan elicitor activity. This activity could be attributed to the charges along the chitin backbone resulting in the formation of multioligomer complexes with membrane components.

Figure 2. Stimulation of chitinase (A) and β-1,3 glucanase (B) activities in grapevine leaves by chitosan with low MW and DDA 80%. Leaves were floated on buffer alone (Control) or in the presence of chitosan at 200 µg/ml, pH 5.9.

Figure 3. Chitosan-induced protection of grapevine leaves against Botrytis cinerea. Excised leaves were pre-treated with chitosan of low MW and DDA 80% at concentrations ranging from 0 to 300 µg/ml for 48 h before challenge with B. cinerea.
The highly active chitosan also induced chitinase and $\beta$-1,3-glucanase activities in grapevine leaves with similar trends (Fig. 2). Both enzyme activities were increased at 10 h after chitosan treatment with maximum accumulation at 24 h, and activity remained high for at least 48 h. Both PR proteins are considered to be functionally implicated in the defense response directed toward chitin and $\beta$-glucans, which are major cell wall components of various fungi.

Because the most active chitosan induced different defense responses that reached their maximum within 48 h, we tested whether it would induce resistance against \textit{Botrytis cinerea} infection. The results show that the pre-treatment of grapevine leaves or plants by chitosan of low MW and DDA 80% conferred a significant protection against the pathogen (Fig. 3). The average necrotic lesion was reduced by about 50% in the 50 $\mu$g/ml chitosan-treated leaves. With 100 to 300 $\mu$g/ml chitosan, this protection reached 60%. The induced protection did not apparently result from any direct effect on pathogen growth but rather from greater induced plant resistance. Application of the same chitosan at concentrations up to 200 $\mu$g/ml to PDA culture medium did not significantly inhibit \textit{B. cinerea} growth. The highest chitosan concentration caused only 10 to 15% inhibition of fungal growth. These data are in agreement with other findings showing that increased resistance to \textit{B. cinerea} was obtained in grapevine plants with high phytoalexin and PR protein levels (Aziz et al., 2003; Jeandet et al., 2002).

We can conclude that chitosan induced an accumulation of stilbene phytoalexins in grapevine leaves depending on its MW and DDA. Highest phytoalexin production was achieved with chitosan of low MW and a DDA of 80%. This chitosan oligomer also activated PR proteins in grapevine leaves. This elicitor capacity of chitosan appeared to be associated with a significant reduction of gray mold disease, which resulted rather from greater induced plant resistance than from direct effect on the pathogen.

**Acknowledgments**

We gratefully acknowledge financial support from Agrolor society and Europôl’Agro.

**References**


Endophytic colonization of grapevine plants by *Burkholderia phytofirmans* strain PsJN enhances host’s growth and resistance to gray mold

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**Abstract**: Although our plant growth promoting bacterium *Burkholderia phytofirmans* strain PsJN has not been isolated from grapevine plants, it is capable of colonization of *Vitis vinifera* L. Upon grapevine inoculation this bacterium is able to establish epiphytic and endophytic populations and improve plant growth as well as resistance to gray mold caused by *Botrytis cinerea*.

**Key words**: PGPB, endophyte, ISR, *Botrytis cinerea*

**Introduction**

Interaction between plants and beneficial bacteria can have a profound effect on plant growth and development, health status, crop yield, and soil quality (Raaijmakers et al., 2002; van Loon & Bakker, 2004; Welbaum et al., 2004; Compan et al., 2005a). Some of these plant growth-promoting bacteria can penetrate plant tissues, and establish endophytic sub-populations within their host (Sturz et al., 2000; Lodewyckx et al., 2002; Compan et al., 2005a). *Burkholderia phytofirmans* strain PsJN (Sessitsch et al., 2005) is one of the best characterized endophytes (reviewed in Nowak & Schulaev, 2003). It was isolated from surface sterilized onion roots and originally identified as *Pseudomonas* sp. strain PsJN (Frommel et al., 1991). This bacterium promotes plant growth, induces systemic resistance, and thrives as endophytes in various plants (Nowak & Schulaev, 2003), including grapevine (Ait Barka et al., 2000, 2002; Compan et al., 2005b, 2006).

**The effect of B. phytofirmans strain PsJN on grapevine growth**

One of the first characteristics of the beneficial interaction between grapevine and *B. phytofirmans* strain PsJN is the enhancement of plant growth. *In vitro* grown grapevine plantlets inoculated with PsJN are sturdier, with better developed and more branched root system, longer stems with more nodes and more leaf hairs when compared to the non-inoculated controls (Ait Barka et al., 2000, 2002; Compan et al., 2005b). Although *in vivo* bacterization of grapevine fruiting cuttings with PsJN has not lead to such remarkable changes as *in vitro*, both on plant morphology and physiology, the bacterium induced earlier flowering in the growth chamber and greenhouse experiments (unpublished results).

**Endophytic colonization**

One other important characteristic of the association between grapevine plants and *B. phytofirmans* strain PsJN is correlated to the endophytic colonization by this bacterium. Using
the wild-type and gfp2x or gusA derivatives of *B. phytofirmans* strain PsJN, we have demonstrated that under gnotobiotic conditions the bacterium colonizes root surface and is able to enter inside roots through lateral root cracks and penetration of root tips and/or by secreting cell-wall degrading enzymes. Once inside roots it can colonize intercellular spaces in several cortical cell layers, break endodermis and enter central cylinder and xylem vessels. Following root colonization, PsJN can then systemically colonize entire plantlets *in vitro*, spreads via xylem vessels with transpiration stream and establishes endophytic sub-populations in leaves, particularly in sub-stomatal chambers (Compant et al., 2005b).

We have also demonstrated that following *in vivo* inoculation of roots the bacterium is able to colonize fruiting cuttings and thrive as endophyte both in root and inflorescence tissues, although its endophytic titer in inflorescences seems to be very low (between 10 and 100 cfu/g FW; unpublished results), probably due to a competition with other endophytic bacteria naturally residing inside grapevine plants.

**Grapevine defence responses**

Following root colonization *B. phytofirmans* strain PsJN induces defence responses in *in vitro* plantlets, such as thickening of the cell wall in cortex cells (Compant et al., 2005b) and accumulation of polyphenols (Compant et al., 2006). Strengthening of the xylem parenchyma cell walls also occurred after colonization (Compant et al., 2006).

The defence responses were also observed after *in vivo* colonization of fruiting cuttings. The experiments with grapevine plants revealed that expression of genes encoding PR proteins can be induced locally, in roots, as well as systemically, in inflorescences, even before that *B. phytofirmans* strain PsJN establishes an endophytic sub-population in the reproductive organs (unpublished results, poster presented at the IOBC meeting).

**Induced systemic resistance**

Following root colonization, *B. phytofirmans* strain PsJN can induced systemic resistance responses in grapevine against gray mold caused by *Botrytis cinerea* as demonstrated after pathogen inoculation of leaves *in vitro* (Ait Barka et al., 2000, 2002) and, more recently, in flowers of the bacterized fruiting cuttings (unpublished results). The PsJN enhancement of the resistance of fruiting cuttings to this fungal pathogen can have practical significance in protecting grapevine flowers against early infections (unpublished results, poster presented at the IOBC meeting), a key step in protection against strong gray mould symptoms development which occur more later in berries (Pezet et al., 2004).

**Conclusions**

Although grapevine is not a natural host for the plant growth promoting bacterium *B. phytofirmans* strain PsJN, as it was originally isolated from onion roots, this plant can be colonized by the bacterium upon inoculation and a beneficial association can occur. The bacterium can colonize various organs endophytically from the roots system to aerial plant parts and the benefits of bacterization include plant growth stimulation and enhanced resistance to gray mold caused by *B. cinerea*. The described isolate PsJN is thus a promising candidate for use in viticulture.

**Acknowledgements**

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References


Biocontrol agents
Ecology
Comparative study of the ecological niche of *Penicillium expansum* Link., *Botrytis cinerea* Pers. and their antagonistic yeasts *Candida oleophila* strain O and *Pichia anomala* strain K

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**Abstract:** The protective level of both yeast strains *Pichia anomala* and *Candida oleophila* against *P. expansum* and *B. cinerea* was previously positively correlated with a yeast density superior or equal 10^4 CFU/cm² of fruit surface in practical conditions. Trials in laboratory conditions confirmed this observation and highlighted that the protective level might depend on the humidity level on fruit surface. A study of the ecological factors (water activity and temperature) susceptible to influence the yeast density on fruit surface was undertaken. *In vitro*, both yeast strains had a similar ecological niche as compared with that of both wound pathogens of apple. Nevertheless at low water activities and low temperatures, the lag time to start antagonistic yeast growth was higher than that observed for wounds pathogens. The *in vivo* study allowed designing two predicting growth models for describing the yeast density on apple fruit surface according to the relative humidity, incubation temperature and the initial concentration of yeast application. A weak yeast density was observed at low relative humidity (75%). The effect of humidity appeared to be more important than that of incubation temperature. For both pathogens, the *in vivo* study revealed only significant effect of incubation temperature on diameter lesion. The comparison between the *in vitro* and *in vivo* trials underlined that yeasts followed the same growth tendency. In contrast, the growth of pathogens was limited at relative humidity close to saturation (98%). All results suggest the importance to maintain the storage room at saturate relative humidity in order to reduce the losses due to blue and grey moulds.

**Keywords:** apples, efficacy, environmental factors, modelling, post-harvest

**Introduction**

*Candida oleophila* strain O and *Pichia anomala* strain K are two antagonistic yeasts isolated from Golden Delicious for their biocontrol activity against post-harvest fungal diseases of apple fruits (Jijakli et al., 1993). Several studies were undertaken to determine the mechanisms of action of both antagonistic yeasts (Jijakli & Lepoivre, 1998; Friel et al., 2004). However, little attention was given to the influence of environmental factors like temperature, water activity and relative humidity on *in vitro* and *in vivo* growth of the antagonistic yeast and their pathogenic target. Such study is crucial to determine optimal and detrimental conditions for fungal infection and to assess the ecological fitness of antagonists. Data obtained will influence the bio-pesticide formulation, in order to favour the antagonistic agent. Lahlali et al. (2005) reported that the *in vitro* growth of *P. expansum* was significantly affected by water activity of medium and temperature. They underlined that the effect of water activity was more important than that of temperature.

The present work focused on two major objectives. On the one hand, the influence of water activity ($a_w$) and temperature was evaluated on the *in vitro* growth rate of both antagonistic strains and both pathogens. On the other hand, similar experimentations were
carried out *in situ* and the establishment of yeast population density on apple fruit surface was evaluated according to the temperature and relative humidity.

**Material and methods**

*Antagonistic strains*

*P. anomala* strain K and *C. oleophila* strain O were cultured at 25°C for three successive generations on Potato Dextrose Agar (PDA) medium with an interval of 24 hours. The final concentrations of both yeasts were adjusted according to D.O. measurement as previously described (Jijakli & Lepoivre, 1998; Jijakli, 1996).

*P. expansum* strain vs2 and *B. cinerea* strain V were isolated from decayed apple fruits by the Plant Pathology Unit (Gembloux Agricultural University, Belgium) and stored onto PDA medium at 4°C in darkroom. The conidial suspension was prepared from 9±2 day-old cultures of pathogen cultivated on PDA medium by scraping the surface of the colonies recovered with Tween 20 (0.05%). Spores concentrations were counted with a Bürker’s cell and these concentrations were adjusted only with sterile distilled water.

*In vitro effect of water activity and temperature on growth rates of post-harvest pathogens of apple fruits and their antagonistic yeasts*

The basic medium used was Potato Dextrose Agar (PDA, \(a_w \approx 0.995\)) with an \(a_w\) of 0.995. The \(a_w\) was modified by adding increasing amounts of glycerol, sorbitol, glucose or NaCl (Lahlali et al., 2006) to obtain \(a_w\) levels of 0.980, 0.960, 0.930, 0.910 and 0.890 at 5, 15 and 25°C. The \(a_w\) of all media was measured with an AquaLab series 3 instrument (Decagon, 950 NE Nelson Court Pullman, Washington 99163). Prepared Petri dishes were inoculated with pathogens (10 µl of a 1×10^6 spores/ml suspensions were deposited in the centre) or antagonist strains (100 µl of 1×10^4 CFU/ml suspensions were plated). Each experiment was carried out in triplicate for each solute-\(a_w\)-temperature combination.

The evaluated parameters were the radial mycelium growth and the CFU/ml respectively for pathogens and antagonistic strains (Lahlali et al., 2005, 2006). The time required for growth (lag phase) was also recorded in each experiment for each treatment. A second-order polynomial equation was used to fit the square root of the radial growth rate and the logarithm of CFU/ml respectively for pathogens and antagonistic yeasts whatever the solute (Lahlali, 2006).

*In vivo effect of relative humidity and temperature on lesion diameters induced by post-harvest pathogens of apple fruits and yeast density*

The different values of relative humidity (75, 86.5 and 98%) inside desiccators were controlled using the saturated salt solutions with respect to studied temperatures (5, 15 and 25°C) (Lahlali, 2006; Xu et al., 2001).

Apple fruits Golden Delicious were disinfected by soaking during two minutes in sodium hypochlorite solution (10%) then rinsed twice in sterile water. For both pathogens, disinfected fruits were wounded in two sites at their equatorial zone with a depth of 4 mm and 1-2 mm in diameter. Each wound was inoculated with a suspension of 10 µl of *P. expansum* and *B. cinerea* at 1×10^6 conidia/ml. The inoculated fruits were placed in desiccators in various environmental conditions according to 3^2 full factorial design with three replicates. After 30 days of incubation, the diameter lesion was measured (Lahlali, 2006).

For both antagonistic yeasts, the disinfected apples fruits were subjected to the Box-Benkenh combination with three replicates and three central points. The initial concentration of yeast application (1×10^4, 5×10^7 and 1×10^8 CFU/ml), the relative humidity (75, 86.5 and 98%) and the temperature (5, 15 and 25°C) were evaluated. After 48 hours of incubation, the
recovery of yeast cell on intact fruit surface was performed as previously described (Lahlali, 2006).

**Results and discussion**

**In vitro effect of water activity and temperature on growth rate of post-harvest patogens of apple fruits and their antagonistic yeasts**

The growth of pathogens and antagonistic strains was significantly influenced by the water activity of medium. All strains showed a growth rate increase with increasing $a_w$ of medium (Lahlali, 2006).

The results showed that *P. expansum*, *B. cinerea*, *C. oleophila* strain O and *P. anomala* strain K had a similar ecological niche. The only difference was observed from *B. cinerea* which was the sole micro-organism unable to grow at $a_w$ of 0.89. Nevertheless at low water activities and low temperatures, the lag time to start antagonistic yeast growth was higher than that observed for wound pathogens (Table 1). These results support the necessity to apply the antagonists as soon as possible after harvest. This application could be considered as an adequate way for pre-colonizing wounded fruits before the arrival of conidial pathogens.

Table 1. Comparative of $a_w$ minimal for growth and the lag time before growth of wounds pathogens and their antagonistic yeasts

<table>
<thead>
<tr>
<th></th>
<th>Minimal $a_w$ value for growth</th>
<th>Lag time at low $a_w$ and temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. expansum</em></td>
<td>0.91-0.93 (NaCl) or $\leq$ 0.89 (non-ionic solutes)</td>
<td>++</td>
</tr>
<tr>
<td><em>B. cinerea</em></td>
<td>0.93 (NaCl) or $&gt;$ 0.89 (non-ionic solutes)</td>
<td>+</td>
</tr>
<tr>
<td><em>P. anomala</em> souche K</td>
<td>0.93 (NaCl) or $\leq$ 0.89 (non-ionic solutes)</td>
<td>-</td>
</tr>
<tr>
<td><em>C. oleophila</em> souche O</td>
<td>0.93 (NaCl) or $\leq$ 0.89 (non-ionic solutes)</td>
<td>-</td>
</tr>
</tbody>
</table>

Non-ionic solutes (sorbitol, glycerol and glucose), +: short, -: long

These *in vitro* results are in agreement with previous findings describing the growth of both yeast strains on apple fruits in three scenarios reflecting the practical conditions (Lahlali and Jijakli, 2004). Yeast population recovered from apple wounds was highest when wounds were performed on wet apples, as compared to wounds performed on dry apple surface. Therefore, the humidity could be considered as a factor limiting the growth of yeasts, especially in pre-harvest conditions.

**In vivo effect of relative humidity and temperature on lesion diameters induced by post-harvest pathogens of apple fruits and yeast density**

The *in vivo* results showed a lower yeast density on apple fruits surface when the antagonistic yeasts were applied at a concentration of $10^4$ CFU/ml for a relative humidity value ranging from 75 to 86.5%, and at temperature situated between 5 and 15°C. The table 2 summarizes the regression coefficients, estimated by multiple regressions analysis. For each antagonistic strain, the variation of population density was mainly explained by the quadratic equation model (93.6% for strain O and 95.8% for strain K). The resulting variation (6.4% for strain O and 4.2% for strain K) remained unexplained by the model equation (Box & Draper, 1987). Whatever the strain, the initial concentration of application had a major and significant effect on the yeast density on apple fruit surface followed respectively by the relative humidity and the temperature.
Table 2. Coefficients significance of Box-Behnken (1960) experimental design adopted for estimating the population density of strain O or strain K on apple fruit surface, obtained after multiple regression analysis.

<table>
<thead>
<tr>
<th></th>
<th>Antagonistic yeast</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain O</td>
<td>Strain K</td>
<td></td>
</tr>
<tr>
<td>Response mean</td>
<td>$\beta_0$</td>
<td>3.57***</td>
<td>3.90***</td>
</tr>
<tr>
<td>T</td>
<td>$\beta_1$</td>
<td>0.65***</td>
<td>0.87*</td>
</tr>
<tr>
<td>RH</td>
<td>$\beta_2$</td>
<td>0.72***</td>
<td>1.02***</td>
</tr>
<tr>
<td>Con</td>
<td>$\beta_3$</td>
<td>1.23***</td>
<td>1.58***</td>
</tr>
<tr>
<td>$T^2$</td>
<td>$\beta_{11}$</td>
<td>-0.046ns</td>
<td>-0.072ns</td>
</tr>
<tr>
<td>RH$^2$</td>
<td>$\beta_{22}$</td>
<td>0.18ns</td>
<td>-0.092ns</td>
</tr>
<tr>
<td>Con$^2$</td>
<td>$\beta_{33}$</td>
<td>-0.92***</td>
<td>-1.13**</td>
</tr>
<tr>
<td>$T \times RH$</td>
<td>$\beta_{12}$</td>
<td>0.31***</td>
<td>-0.02***</td>
</tr>
<tr>
<td>$T \times Con$</td>
<td>$\beta_{13}$</td>
<td>-0.11ns</td>
<td>-0.23ns</td>
</tr>
<tr>
<td>RH$ \times$ Con</td>
<td>$\beta_{23}$</td>
<td>-0.20*</td>
<td>-0.30**</td>
</tr>
</tbody>
</table>

T: temperature; RH: relative humidity and Con: initial concentration of application. *** Highly significant; * Significant.

Focusing on both pathogens, it appeared that incubation temperature had a significant impact on the development of lesion diameter. In opposite, there was no significant effect of relative humidity. The part of variation explained by both studied factors is 52 and 55% respectively for *P. expansum* and *B. cinerea*. The highest lesion diameters were observed at relative humidity of 86.5%.

Table 3. Coefficients significance of the $3^2$ full factorial adopted to study the combined effect of relative humidity and incubation temperature on the lesion diameter of *P. expansum* and *B. cinerea*.

<table>
<thead>
<tr>
<th></th>
<th>Pathogenic fungi</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. expansum</em></td>
<td>B. cinerea</td>
<td></td>
</tr>
<tr>
<td>Response mean</td>
<td>$\beta_0$</td>
<td>34.66*</td>
<td>27.23*</td>
</tr>
<tr>
<td>T</td>
<td>$\beta_1$</td>
<td>3.96*</td>
<td>3.28*</td>
</tr>
<tr>
<td>HR</td>
<td>$\beta_2$</td>
<td>1.06ns</td>
<td>-0.51ns</td>
</tr>
<tr>
<td>$T^2$</td>
<td>$\beta_{11}$</td>
<td>-0.30ns</td>
<td>-4.27ns</td>
</tr>
<tr>
<td>HR$^2$</td>
<td>$\beta_{22}$</td>
<td>-7.79*</td>
<td>-11.08*</td>
</tr>
<tr>
<td>$T \times HR$</td>
<td>$\beta_{12}$</td>
<td>2.71ns</td>
<td>0.56ns</td>
</tr>
</tbody>
</table>

* Significant

Both antagonistic yeasts presented similar behaviour during *in vitro* and *in situ* experimentations. In the opposite, the growth of pathogens was highly influenced by water activity during *in vitro* experimentations and by temperature during *in vivo* conditions. This result can indicate that the relative humidity of the wound inside fruits is sufficient to support the growth of both fungi, suggesting that after infection, the progress of the decay will not be limited by the variation of relative humidity. The *in vivo* growth of pathogens was also reduced at relative humidity close to saturation (98%) as compared to relative humidity
ranging from 80 to 92%. All results support the importance to maintain the storage room at saturate relative humidity in order to reduce the losses dues to blue and green moulds and to create the optimal conditions of antagonistic yeasts proliferation.

Acknowledgment

This research work was founded by the ‘Agence Universitaire de la Francophonie’ and the Plant Pathology Unit of Gembloux Agricultural University (Belgium) that the authors are grateful.

References


Environmental adaptation of *Pichia anomala* WRL-076 as an effective biocontrol agent for pre-harvest application

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**Abstract:** In laboratory experiments, PEG (polyethylene glycol) 8000 was used to adjust medium $a_w$ to 0.96, which mimicked a water stress condition of $-5.62$ MPa. *P. anomala* WRL-076 can grow at this low water activity ($a_w$). The yeast cells formed a film and inhibited the growth of *A. flavus* inoculated to the medium. Two experiments were conducted in a commercial orchard in the summer of 2005. *P. anomala* WRL-076 reduced the frequency of *A. flavus* colonization by 4 to 10 times and decreased the total propagules of *A. flavus* by 80 to 99% in comparison to nut-fruits not sprayed with the yeast.

**Key words:** biological control, *Aspergillus flavus*, yeast, aflatoxin, pistachio, almond

**Introduction**

There is no conventional fungicide to control the fungus *Aspergillus flavus*. That fungus produces aflatoxin which is the most potent carcinogen known. This mycotoxin is very hazardous to the health of both human and animal. Economic losses are in the billions of dollars per year due to aflatoxin contamination of agricultural commodities. Aflatoxin contamination is associated with wounding in corn, peanut, cotton seed and tree nut (Diener et al., 1987; Cotty et al., 1994; Hua et al., 1998; Hua, 2004, 2006). Numerous literatures indicate that wounds in plant tissues including insect provide the entry to *A. flavus*. By mechanically wounding pistachio nut-fruits, sufficient number of nut-fruits conducive to *A. flavus* and fungal infection were generated. Growers and processors are looking for effective means to control *A. flavus* infestations and subsequent contamination of food crops.

A bioassay has been developed to screen for effective yeast inhibiting both the growth of the *A. flavus* and the aflatoxin production (Hua et al., 1999). Among several hundred yeast isolates tested, *Pichia anomala* strain WRL-076 was selected for its antagonistic activities to reduce conidia production of *A. flavus* and aflatoxin. Experiments were carried out in lab situation using both sterilized and non-sterilized plant materials, i.e., pistachio flowers and nut-fruits as well as in almond and pistachio leaves. Conidia production of *A. flavus* was reduced from 60 to 80% on plant samples sprayed with *P. anomala* WRL-076 compared to the control samples without the spray of this yeast (Hua et al., 1998, 2000; Hua, 2002, 2003). Furthermore in laboratory experiments, PEG (polyethylene glycol) 8000 was used to adjust medium $a_w$ to 0.96, which mimicked a water stress condition of $-5.62$ MPa. *P. anomala* WRL-076 can grow at this low water activity ($a_w$). The yeast cells formed a film and inhibited the growth of *A. flavus* inoculated to the medium. Analysis of cellular trehalose accumulation and genes responsible for trehalose biosynthesis are in progress. We report here the efficacy of *P. anomala* WRL-076 to control *A. flavus* in a commercial pistachio orchard.
Materials and methods

Experimental design
Two experiments were conducted in a commercial orchard. Nut-fruits of pistachio were individually wounded with a dissecting needle. Four treatments were applied: W- sprayed with water; Y- sprayed with an aqueous suspension of yeasts at $5 \times 10^7$ cells/ml; Y+AF- sprayed with an aqueous suspension of yeasts at $5 \times 10^7$ cells/ml and two hours later sprayed with spore suspension of A. flavus at $1 \times 10^3$ cells/ml; AF- sprayed with spore suspension of A. flavus at $1 \times 10^3$ cells/ml. The wounded nut-fruits were harvested on 08/11/05 and 08/31/05 respectively for Exp. 1 (06-30 to 08/11/05) and Exp. 2 (8/11 to /08/30/05).

Sample collection
Wounded pistachio nut-fruits were hand picked from the tree and immediately placed to test tubes (five nuts per tube). The samples were brought back to the lab for analysis. Six hundred nut-fruits from each treatment were analysed in both experiment 1 and 2 to provide sufficient replications for statistical analysis.

Colony forming unit
Microorganisms kept in the tubes were eluted from nut-fruits in Tween 80 solution from the experimental samples by shaking and sonicating. Viable counts of yeast cells and A. flavus propagules were determined by spreading the microorganisms on dichloran rose bengal chloramphenicol (DRBC) agar plate using a spiral plating system (Spiral Biotech Autoplate 4000).

Statistical analysis
Duncan’s multiple range test was used for statistical analysis at $\alpha = 0.05$, data with the same letter are not significantly different.

Results and discussion

Effect of yeast on colonization of wounded pistachio
Samples showed positive for the presence of A. flavus on DRBC agar plates are counted as colonized. In Exp. 1, colonization of nut fruits by A. flavus was 16% for AF treatment, 1.8% for Y+AF. No colonization was observed for nut-fruits sprayed with water and yeast (Fig. 1). In Exp. 2 (Fig. 2), the percent colonization of A. flavus on nut-fruits was 56.8% for AF treatment, 15% for Y+AF treatment, 1.7% for Y treatment and 15% for W treatment. Higher colonization of A. flavus in Exp. 2 than Exp. 1 may be due to a higher level of population of A. flavus naturally present in the orchard in August than in July. These results demonstrate that the yeast, P. anomala, significantly decreased the frequency of colonization by A. flavus in wounded pistachios when sprayed with the biocontrol yeast.

Inhibition of A. flavus propagules production by P. anomala
The propagules of A. flavus from harvested samples were extracted and spread on DRBC plates to determine the CFU (colony forming unit). Figure 3 shows the data for the total number of propagules in the four treatments of Exp. 1. The results indicate that the CFUs of A. flavus in nut-fruits sprayed with AF are 40 times more than the nut-fruits treated with Y+AF. It equivalents to 98% reduction of A. flavus population in the orchards. Similar results were observed in the second experiment (Exp. 2). By comparing water sprayed nut-fruits to yeast sprayed ones, A. flavus population was reduced 97% by the yeast treatment. As shown in Fig. 4, a 75% reduction of A. flavus is accounted due to the spray of P. anomala.

There is no conventional fungicide to control A. flavus. Aflatoxin continues to be a regulatory deterrent to the almond and pistachio industries. Growers and processors are
looking for effective means to control *Aspergillus* infestations and subsequent contamination of edible nuts with aflatoxin. *P. anomala* WRL-076 is not known to produce any toxic compounds or causes disease in plants and animals and humans. The cost of spraying yeast in orchards should be comparable with most other biopesticide. *P. anomala* WRL-076 has been tested as a biopesticide to control *A. flavus* and aflatoxin on corn in Texas. Researchers in California are testing this biopesticide to control *Alternaria* disease in almond and pistachio. Once field efficacy of the yeast has been demonstrated, growers will most likely use it to control *A. flavus* and possibly other fungal pathogens. Application of this pesticide to food crops may result in economic gain to the growers and industries.

![Colonization of *A. flavus* on nuts](image1)

**Figure 1.** Colonization of *A. flavus* on wounded nut-fruits in exp. 1 (06/30/05 to 08/11/05). Ducan’s multiple range test was used, Same letter indicating no significant difference.

![Colonization of *A. flavus* on Nuts](image2)

**Figure 2.** Colonization of *A. flavus* on wounded nut-fruits in exp. 2 (08/11/05 to 08/30/05). Ducan’s multiple range test was used, Same letter indicating no significant difference.

![Average propagules of *A. flavus* on nut-fruit](image3)

**Figure 3.** Growth inhibition of *A. flavus* by *P. anomala*. Exp. 1 (06/30/05 to 08/11/05). Ducan’s multiple range test was used, Same letter indicating no significant difference.
Figure 4. Growth inhibition of *A. flavus* by *P. anomala*. Exp. 2 (08/11/05 to 08/30/05). Duncan’s multiple range test was used, Same letter indicating no significant difference.

**References**


Role of *Ampelomyces quisqualis* on grapevine powdery mildew in Trentino (northern Italy) vineyards

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**Abstract:** Sulphur is the only effective alternative to chemical pesticides against powdery mildew in organic agriculture. However, it is also under criticism in some production systems, as in Trentino (northern Italy), where tourism intermingles human presence and vineyards, and because its negative side effects on beneficials. A natural occurring powdery mildew hyperparasite, *Ampelomyces quisqualis*, is considered as alternatives to chemicals. During two years we monitored the natural presence of *A. quisqualis* strains in Trentino province. Low natural presence of the *A. quisqualis* (less than 1%) was found. It was found as mycelium parasitizing *E. necator* cleistothecia and as conidia, both in conventionally treated and organic vineyards. Among the isolated *Ampelomyces* spp. from the vineyards, a likely new morphotype was identified. Moreover, the efficacy greenhouse and field trials of *A. quisqualis* based bio-fungicide (AQ10) showed partial effectiveness. Under field conditions AQ10 treatments sprayed early in the season (spring) were slightly effective in controlling powdery mildew, on leaves and on grapes, whilst AQ10 was equivalent to sulphur when applied in the last part of the season (late summer). AQ10 sprayed in autumn to reduce over-wintering inoculum was ineffective in reducing powdery mildew in the following season.

**Key words:** biocontrol agent, Erysiphe necator, mycoparasite, organic agriculture

**Introduction**

Powdery mildew, caused by *Erysiphe necator*, is one of the most important grapevine diseases worldwide. At present grapevine protection against powdery mildew is mainly based on the use of chemical fungicides (Hewitt, 1998). To reduce the use of pesticides, several environmental friendly products have been evaluated for controlling this pathogen (Kiss, 2003). Sulphur is at present the only effective alternative in organic agriculture. In areas where tourism intermingles with agriculture, as in Trentino province (northern Italy), and in organic viticulture, the use of sulphur is under discussion.

A natural occurring hyperparasite, *Ampelomyces quisqualis*, is considered one of the best alternatives to sulphur or synthetic pesticides against powdery mildews (Kiss et al., 2004). Several studies showed that *A. quisqualis* can parasitize over-wintering *E. necator* cleistothecia on the bark and that *Ampelomyces* infects and produces picnidia only inside the young cleistothecia (Falk et al., 1995). Until now little is known about the natural occurrence of *A. quisqualis* in Trentino province (Northern Italy), even if grapevine is one of the two most important crops in the area and several treatments are yearly applied. In Trentino “flag shoots” sporadically occur, so it is assumed that *E. necator* cleistothecia on the bark are the main source of inoculum for primary infections in the vineyard.

The main objectives of the present work were to estimate the natural presence of *Ampelomyces* spp. as mycoparasite in the vineyards, to isolate local strains of *Ampelomyces*
spp. for a potential development as biocontrol agents and to evaluate the efficacy of a commercial preparation of *A. quisqualis* (AQ10) used in strategies combined with sulphur in controlling disease and in suppressing pathogen by cleistothecia colonization.

**Material and methods**

The natural presence of *Ampelomyces* spp. as *E. necator* hyperparasite was monitored on leaves and bark in twenty vineyards in Trentino province (northern Italy) in 2004 and 2005, with the method reported by Angeli et al. (2005). Wild strains of *A. quisqualis* were isolated from parasitized cleistothecia collected in the vineyard on potato dextrose agar (PDA, Sigma).

The efficacy of the commercial product AQ10, based on *A. quisqualis* (Intrachem Bio, Italy) was evaluated under greenhouse control conditions and in field trials carried out in 2004 and 2005. Plants of the susceptible variety Pinot Gris, having one shoot with 5-6 green fully expanded leaves, grown under controlled conditions (25±3°C and 50-60% relative humidity.) in a pathogen free greenhouse, were inoculated by shaking powdery mildew infected leaves above plants. Plants were treated twice (6 hours before and 7 days after inoculation) with a ten times higher AQ10 dosage compared to the field (0.6 g/l, 20 ml/plant). Sulphur (Thiovit, Syngenta, 3 g/l) and water treatment were used as references. Each treatment consisted of five plants (replicates).

The field trial was performed in an experimental organic vineyard located in S. Michele all’Adige (Trentino). A highly powdery mildew susceptible variety (Schiava) was used. AQ10 (60 g/ha) and sulphur (3 kg/ha) applications were done with an atomizer (10 hl/ha) at seven days interval. Five different strategies using sulphur and AQ10 were compared (Figure 1). AQ10 was applied when temperature and relative humidity were suitable to the organism (beginning and end of the growing season) and sulphur in the remaining periods. AQ10 late treatments were sprayed two times (7 days interval) during autumn, in order to reduce the source of inoculum (over-wintering cleistothecia) for the next growing season. The assessments were done weekly by randomly checking 100 leaves and 50 bunches in five replicate plots (10 plants each). Incidence (percentage of leaves or bunches with symptoms) and severity (percentage of symptomatic leave and bunch area). Area Under the Disease Progress Curve (AUDPC) was calculated. Analysis of variance (ANOVA) was applied on “arcsin” transformed data, using the software Statistica 7 (Statsoft, Italy). Significant differences among treatments were separated by Duncan’s test.

<table>
<thead>
<tr>
<th>Strategy</th>
<th>May</th>
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<tr>
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<td></td>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>Sulphur</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>(8)</td>
<td>AQ10</td>
<td>(8)</td>
<td>Sulphur</td>
<td>(8)</td>
<td>AQ10</td>
</tr>
<tr>
<td>4</td>
<td>(16)</td>
<td>Sulphur</td>
<td></td>
<td></td>
<td>(8)</td>
<td>AQ10</td>
</tr>
<tr>
<td>5</td>
<td>(12)</td>
<td>Sulphur</td>
<td></td>
<td></td>
<td>(12)</td>
<td>AQ10</td>
</tr>
</tbody>
</table>

Figure 1. Strategies applied in field experiments to control powdery mildew in organic viticulture in Trentino in 2005. Numbers in brackets indicate the number of application made.

**Results and discussion**

The two year monitoring underlines a low natural presence of *Ampelomyces* spp. in Trentino vineyards (less than 1%). *Ampelomyces* spp. were found only on leaves, as mycelium
parasitizing *E. necator* cleistothecia and as conidia, both in conventionally treated and organic vineyards. No parasitized cleistothecia were found on the bark. Among the isolated *Ampelomyces* spp. from the vineyards, a likely new strain was identified, differing in conidia shape from the commercial strain (AQ10). The conidia of the wild isolate are more fusiform, compared to the ellipsoidal ones of AQ10 (Fig. 2).

![Figure 2. Differences in shape of Ampelomyces spp conidia between the wild strain (left) and the commercial ones (right)](image)

The wild-type *Ampelomyces* sp. was isolated on PDA, but developed well in different nutrient substrates (CZEPEK, MYEA), showing better growth rate on malt extract agar and PDA. *In vitro* on PDA at 25°C the *Ampelomyces* sp. isolate forms mycelium within 5-7 days and produces both picnidia and conidia after 18-20 days.

In greenhouse trials powdery mildew severity did not significantly differ in *A. quisqualis* and untreated plants. This may be due to the low RH and lack of water on leaves that is important for *A. quisqualis* germination or because the pathogen was not sufficiently established when the hyperparasite was applied.

In the vineyard during 2005 weather conditions were suitable to *E. necator*. Under high disease pressure (incidence on untreated bunches was 100%) integrated strategies using AQ10 with sulphur reduced powdery mildew symptoms compared to the untreated control. *A. quisqualis* applied early during spring in strategy 3 was less effective than strategy 2 which applies sulphur (Fig. 3). No difference with sulphur was found in the strategies that applied AQ10 at the end of the season. In the treated vineyard, at the end of the season only 1% of cleistothecia collected from leaves were parasitized by *A. quisqualis* with no differences among the strategies. AQ10 late treatments had no effect on powdery mildew infections in the following growing season. At the end of June, on AQ10 treated and untreated bunches powdery mildew incidence was respectively 35 and 38%.

In conclusion, during the last two years the natural presence of *Ampelomyces* spp. in Trentino was very low. This can be related to the low level of disease that was present in the previous five years in the area, which reduced the natural population of the hyperparasite or to prohibitive climatic condition for *A. quisqualis* development. The efficacy of AQ10 on leaves in controlled conditions was insufficient. On bunches the use of AQ10 at the beginning of the season had a low effectiveness, but it was more effective if applied at the end of the season. The low level of parasitisation of *E. necator* cleistothecia in the AQ10 treated vineyard could
be related to unsuitable environmental condition for the commercial *A. quisqualis* strain. If the low efficacy of AQ10 is related to unsuitable environmental conditions of the studied area, the wild *Ampelomyces* spp. isolate naturally present in the vineyard could be an interesting microorganism to be further studied.

![Figure 3](image-url)  
Figure 3. Powdery mildew severity (AUDPC index) on bunches during 2005 season. Columns with same letter do not differ with $P \leq 0.05$ (Duncan’s test).

**Acknowledgements**

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Molecular ecology of bacterial and fungal *Verticillium* antagonists in/on different host plants and soils

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**Abstract:** To study the effect of plant species and soil type on the proportion and diversity of naturally occurring antagonistic bacteria and fungi towards *Verticillium dahliae* Kleb., microorganisms isolated from oilseed rape and strawberry rhizospheres were analyzed and compared to those retrieved from bulk soils. Samplings took place on randomised field trials at three different locations in Germany over two growing seasons. Each of the investigated microbial community harboured a high proportion and a broad spectrum of *Verticillium* antagonists. The proportion and composition of antagonists was influenced by the plant species and by the site. Bacterial antagonists showed a higher degree of plant specificity than fungal ones. Altogether, a large list of new *Verticillium* antagonists was detected. The latter and the knowledge about naturally occurring antagonists can be translated into new concepts to control *Verticillium*.

**Keywords:** verticillium wilt, biocontrol

**Introduction**

One of the most important soil-borne pathogens is *Verticillium dahliae* Kleb., causing Verticillium wilt responsible for high yield losses in a wide variety of host plants including many important crops such as strawberry and oilseed rape. With the impending phase-out of the fumigant methyl bromide worldwide, there is no possibility to suppress the pathogen, and therefore alternative management strategies are required. One of these alternatives is the use of plant-beneficial microorganisms which can suppress soil-borne pathogens in the rhizosphere. The rhizosphere is defined as the layer of soil influenced by root metabolism. In comparison to root-free soil, the rhizosphere forms a nutrient-rich niche for microorganisms as a result of exudation of compounds. Antagonistic activities of numerous microbial populations in the rhizosphere influence plant growth and health.

In a previous three-year field study we analyzed the effects of plant species on the rhizosphere-associated bacterial communities and on bacterial antagonists from *Verticillium* host plants in comparison to bulk soil. The proportion and composition of bacterial antagonists in the rhizospheres of potato, oilseed rape and strawberry was shown to be influenced by the plant species and growth stage (Berg et al., 2002). The strawberry rhizosphere was characterized by a high proportion and a low diversity of antagonists. In contrast, a low proportion and a high diversity of bacterial *Verticillium* antagonists were
observed for the rhizosphere of oilseed rape and potato. Plant specificity of the rhizosphere-associated bacterial communities was also shown using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes amplified from community DNA (Smalla et al., 2001). DGGE patterns of oilseed rape and potato rhizosphere communities were more similar to each other than to the strawberry pattern. Several studies have also analyzed the effect of soil and plant type on bacteria (rev. in Garbeva et al., 2004). However, knowledge about the effect of both factors on the antagonistic community is missing, and was therefore the objective of this study.

Material and methods

Experimental design
Two crop plants, oilseed rape (*Brassica napus* L. [family: *Brassicaceae*]) cv. Licosmos and strawberry (*Fragaria x ananassa* [Duchense] Decaisne & Naudin [family: *Rosaceae*]) cv. Elsanta were grown in a randomized block design with four replicates per crop plant during two vegetation periods (2002, 2003). The fields for the trials were located in Berlin (52°31’N, 13°24’E), Braunschweig (52°16’N, 10°31’E), and Rostock (54°05’N, 12°07’E). Soil parameters are described in Berg et al. (2005).

Isolation of bacterial and fungal strains and determination of CFU
Plant roots with adhering soil taken from five or more plants per plot were pooled into sterile Stomacher bags and treated as one sample. Prior to cell extraction, 5 g of each pooled sample were transferred into a new Stomacher bag and treated as described in Berg et al. (2005). For each sample the suspensions were serially diluted and plated onto Saltwater Nutrient Agar or SNA (fungi) and R2A (bacteria). In two consecutive years a total of 240 bacterial and fungal strains per site and sampling time were randomly selected and screened for antagonistic activity.

Screening for bacteria and fungi antagonistic to *Verticillium dahliae*
The *in vitro* inhibition of *Verticillium dahliae* Kleb. was determined in a dual culture assay on Waksman agar (WA) according to Berg et al. (2002). All strains were tested in three independent replicates with *Verticillium dahliae* V25 (isolated from *Brassica napus* L.) and *Verticillium dahliae* V35 (isolated from *Fragaria x ananassa* [Duchense] Decaisne & Naudin). Zones of inhibition were measured after 3 to 7 days of incubation at 20°C.

Identification of antagonistic strains
All fungal strains were characterized morphologically and identified by 18S rRNA gene sequencing. The bacterial strains were first characterized using ARDRA, and according to the genotypical groups representatives were analyzed by 16S rRNA gene sequencing. Furthermore, BOX-PCR fingerprints were generated for representative strains. The genotypic diversity of the bacterial communities was measured by the Shannon information theory function (Shannon & Weaver, 1949).

Statistics
All data (CFU, % of *Verticillium* antagonists, diversity indices) were analyzed for significance using U-Test "Mann-Whitney" (P≤0.05) and studied by two-factor analysis of variance by Statistical Product and Service Solutions for Windows, Rel. 9. 0. 1. (SPSS Inc., Chicago, Illinois). BOX-PCR generated fingerprints were evaluated computer-assisted by the GelCompar program of Applied Maths (Version 4.1, Kortrijk, Belgium).
Results and discussion

Isolation of fungi and bacteria from the rhizospheres and from soil
Altogether, CFU counts on SNA were in the range of $\log_{10} 3.9$ CFU g$^{-1}$ and 5.9 g$^{-1}$ root fresh weight [rfw] (average $\log_{10} 4.8$) while CFU counts on R2A ranged from $\log_{10} 7.5$ CFU g$^{-1}$ to 8.5 g$^{-1}$ (average $\log_{10} 8.0$). Enhanced abundances in both rhizospheres in comparison to bulk soil were only determined for bacteria; for fungi no rhizosphere effect was shown. No statistically significant differences were found between plant species and sites.

Screening for isolates antagonistic to Verticillium dahliae
Altogether, 4,320 fungal isolates were screened for their ability to suppress $V. dahliae$ in an in vitro dual culture assay. Initially 911 isolates were found which were active against $V. dahliae$. Although similar numbers of isolates per site were tested, the proportion of isolates with antagonistic activity was slightly different. The highest proportion on average was found for Braunschweig (27%) followed by Rostock (25%) and Berlin (21%). The proportion of isolates with antifungal activity was higher for bulk soil (27%) than for both rhizospheres (22%). However, differences between the sites and the microenvironments were not statistically significant. In general the proportion of bacterial antagonists retrieved from rhizosphere and bulk soils was lower in comparison to the fungi. From R2A agar, 341 (7.7%) bacterial isolates were found which suppress $Verticillium$ growth by inhibition of mycelium or microsclerotia formation. The proportion of antagonistic isolates was rather different for each site and microenvironment. Their proportion varied between 4 and 13% and was highest on average for Braunschweig (11%) followed by Berlin (9.8%), and Rostock (7.8%). Again, the proportion of isolates with antifungal activity was significantly lower for bulk soil (4.2%) than for both rhizospheres (9.6%). A higher proportion of antagonists was found on average in the strawberry rhizosphere (10.2%) than in the rhizosphere of oilseed rape (8.9%) although this difference was statistically not significant. For bacterial as well as fungal antagonists a seasonal shift has been detected.

Diversity of antagonistic strains
The morphological characterization of 911 fungal $Verticillium$ antagonists resulted in 26 morphological groups. Out of them, 76 morphotypes were represented by single isolates. The highest number of morphotypes was found in Rostock (54 clusters: 15 groups and 39 single isolates) and in bulk soil (53 clusters: 19 groups and 34 single isolates). $Penicillium$, $Trichoderma$, $Monographella$ and $Paecilomyces$ were the most dominant genera. Altogether, a plant- and site-dependent composition and genotypic diversity of antagonistic morphotypes was found. The strawberry rhizosphere was characterized by a preferential occurrence of $Penicillium$ and $Paecilomyces$ isolates, a low number of morphotypes (31) and species (13) while $Monographella$ isolates were most frequently obtained from the rhizosphere of oilseed rape for which a higher number of morphotypes (41) and species (17) was found. $Trichoderma$ strains displayed a high diversity in all soils but a high degree of plant specificity was shown by BOX-PCR fingerprints. The diversity of rhizosphere-associated antagonists was lower than in bulk soil suggesting that some fungi were specifically enriched in each rhizosphere. In respect to the antagonistic bacteria, a total of 560 active isolates was analyzed among which $Pseudomonas$ (77%), $Serratia$ (6%) and $Streptomyces$ (5%) were the most dominant genera. The rhizosphere effect, only shown for the antagonistic bacterial populations, was shown by an enhanced proportion of antagonistic isolates, by enrichment of specific ARDRA types, species and genotypes as evidenced by BOX-PCR, and by a reduced diversity of antagonistic bacteria in the rhizosphere in comparison to bulk soil. Such an effect was influenced by the plant species and by the site of its cultivation. Altogether, 16S rRNA gene sequencing of 66 isolates resulted in the identification of 22 different bacterial species.
Antagonists of the genus *Serratia* were preferentially isolated from oilseed rape rhizosphere, with the exception of one site. For isolates of *Pseudomonas* and *Serratia* plant-specific and site-specific genotypes were also found. Interestingly, these findings were underlined by a study analysing the *Pseudomonas* communities in both oilseed-rape and strawberry rhizospheres by means of cultivation-independent techniques (Costa et al., 2006). A model showing the selective enrichment of antagonists in the rhizosphere is shown in Fig. 1.

**Implications for biocontrol**

The indigenous antagonistic potential is important for plant growth and health. In each of the investigated rhizospheres potentially antagonistic populations against the soil-borne pathogen *V. dahliae* were found. Surprisingly, the proportion of fungal antagonists was higher in general, and consists of 1/3 of the culturable fungal population. All antagonistic communities showed a high diversity. However, ubiquitously-occurring genera such as *Pseudomonas*, *Penicillium* and *Trichoderma*, and specialized antagonists like *Serratia* and *Paecilomyces* were found. Depending on the ecological behaviour of the pathogen that needs to be controlled, antagonists from both groups can be selected. Plant specificity of antagonists may have an influence on the composition of the indigenous microflora in soil, and might explain well-known phenomena such as soil fatigue and suppressiveness. Therefore, the importance of land management strategies such as crop rotation must be underlined.

<table>
<thead>
<tr>
<th>Rhizosphere of strawberry</th>
<th>Rhizosphere of oilseed rape</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Verticillium</em> antagonists: high</td>
<td><em>Verticillium</em> antagonists: lower</td>
</tr>
<tr>
<td>10.2% bacteria; 22% fungi</td>
<td>8.9% bacteria; 22% fungi</td>
</tr>
<tr>
<td>Diversity (species, genotype): low</td>
<td>Diversity (species, genotype): high</td>
</tr>
<tr>
<td>Characteristic genera:</td>
<td>Characteristic genera:</td>
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<td><em>Pseudomonas</em> and <em>Streptomyces</em></td>
<td><em>Pseudomonas</em> and <em>Serratia</em></td>
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<td><em>Penicillium</em> and <em>Paecilomyces</em></td>
<td><em>Penicillium</em> and <em>Monographella</em></td>
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</table>

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**Rhizosphere effect: Selective enrichment of antagonist**

- % *Verticillium* antagonists enhanced for bacteria
- % *Verticillium* antagonists constant for fungi
- Reduced diversity at pheno- and genotypic level

**Soil as reservoir for *Verticillium* antagonists**

- Low proportion of bacterial and high of fungal antagonists
- High diversity at pheno- and genotypic level

Figure 1. Model for the selective enrichment of antagonists in the rhizosphere

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electrophoresis of PCR-amplified fragments of 16S rRNA genes - plant-dependent
Phyllosphere microbial communities in pot roses respond to low P fertilization and mycorrhiza inoculation, but not to application with the biocontrol fungus *Ulocladium atrum*

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**Abstract:** Biocontrol of grey mould in pot roses by combined inoculation with the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* and the biocontrol fungus *Ulocladium atrum* was examined in a greenhouse pot experiment with low (5 ppm) and conventional phosphorous (50 ppm) provided with the nutrient solution. The experiment had a fully factorial design with three main factors: 1) *G. mosseae* (±), 2) *U. atrum* (±) and 3) phosphorus (P) fertilization (5 and 50 ppm). Each of the eight treatments had five replicates each with five pots and each pot had three plants. Rose cuttings were rooted in a peat-based substrate, which in the treatments with mycorrhiza were mixed with inoculum of *G. mosseae*. Plants were trimmed to five cm above the surface five and nine weeks after rooting. Immediately after the second trimming spores of *U. atrum* was applied by spraying a water suspension with $10^6$ spores per plant. The following day the same amount of spores of *Botrytis cinerea* were applied to the foliage. Plants were harvested two weeks after *B. cinerea* application.

At harvest plants were scored for grey mould development by counting lesions and also shoot and root dry weights were measured. Furthermore, the microbial communities in the phyllosphere were examined by incubating three leaves from each plant in 1/10 tryptic soy broth for 24 hrs. Microorganisms from the enrichment culture were extracted by centrifugation and the pellet was subjected to whole cell fatty acid analysis.

Overall shoot dry weight was highest in treatments with conventional P. Inoculation with *G. mosseae* increased shoot dry weight in combination with low P, whereas *U. atrum* had no effect on plant growth. Low P and *U. atrum* markedly reduced grey mould development, whereas mycorrhiza had no influence on grey mould. Phyllosphere microbial communities responded to low P and interactions between mycorrhiza and low P were found, whereas *U. atrum* had no effect.

In conclusion, combined inoculation with *G. mosseae* and *U. atrum*, especially at low P fertilization, seems to be a promising strategy to manage grey mould in production of pot roses. Our results indicate that changes in the phyllosphere microbial communities, may be involved in the control of grey mould in the low P treatment, but most likely not in grey mould control with *U. atrum*. 
Root application of bacterial antagonists to field-grown lettuce: effects on disease suppression and non-target microorganisms

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Abstract: The phytopathogenic fungus Rhizoctonia solani causes high yield losses in agricultural and horticultural crops. As no effective control is available in organic farming or horticulture, biological control using naturally antagonistic bacteria can supply new control strategies. After several in vivo and in vitro tests the two rhizobacteria Pseudomonas trivialis (3Re2-7) and Pseudomonas fluorescens (L13-6-12) as well as the potato endophyt Serratia plymuthica (3Re4-18) were selected as effective Rhizoctonia antagonists. In our study the biocontrol effect and the impact of these antagonistic bacteria on non-target lettuce-associated microorganisms was assessed after their root application in two field trials in Germany. The biocontrol effect of all introduced bacteria, which includes a significant increase of the dry weight and a significant decrease of the disease severity, could be shown for the first time in a field trial. The culture-independent method of PCR-single-strand conformation polymorphism (SSCP) analysis was used to examine the microbial communities of the rhizosphere, the endorhiza and the endophyllosphere of field-grown lettuce. Primers targeting the ITS1 region or highly variable regions of the 16S rDNA were applied to obtain fingerprints of the fungal or bacterial communities, respectively. As expected, SSCP fingerprints of the lettuce-associated microbial communities revealed a much higher bacterial and fungal diversity in the rhizosphere than in the endophytic microhabitats. So far, only transient changes in the composition of the bacterial communities were found. Comparison of SSCP fingerprints revealed a high dependence of community composition on field site, plant growth stage and microenvironment, while no long-term impact could be found due to the bacterial treatments. Generally, only little is known about the microbial communities associated with field-grown lettuce. Therefore, DNA bands of the SSCP fingerprints, representing the dominant members of the lettuce-associated microbial communities, were further characterised by cloning and sequencing. Next to various unspecified uncultured species, the sequences of SSCP bands matched NCBI database entries of the fungal genera Cladosporium, Trichoderma, Tetracladium and Bremia as well as the bacterial genera Pseudomonas, Variovorax, Staphylococcus and Rhodanobacter. Based on the results, an environmentally friendly and efficient biocontrol strategy can be developed.
Effect of introduced epiphytic yeast on an insect pest
(Cydia pomonella L.), on apple pathogens (Venturia inaequalis and
Podosphaera leucotricha) and on the phylloplane chemical
composition

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Abstract: Research on biocontrol agents (BCAs) such as yeasts, bacteria and fungi against plant
pathogens is developing fast, but the effects of the introduction of these microorganisms on non target
organisms and on the plant host physiology are poorly known. We study the effects of the spray of an
epiphytic yeast (Y16), a potential biocontrol agent against the powdery mildew, on scab, the codling
moth and the phylloplane chemical composition. A change in the codling moth egg quantity laid on
the apple tree treated with the yeast suspension was strong. In the second season of experiment, during
the second flight period, we observed an increase of the egg laid on the treated tree, especially on tree
site close to the apple fruits. These results are however, in contradiction with the preliminary
experiment. A third season will be conducted this summer in order to clarify the effect of the yeast on
the quantity of egg laid. Several yeast and bacterium isolates were tested for their ability to control
apple powdery mildew in the frame of a screening program. The yeast Y16 showed a good potential as
BCA of this disease. Three weeks from the inoculation, disease severity (percentage of leaf area
covered by symptoms) in the water control was 37.4%. The severity of the yeast treated plants was
reduced to 7.6%. We are currently analysing the effect of the yeast suspension on the germination of
V. inaequalis conidia. Primary metabolites (sugars) of the phylloplane stimulate the egg laying of the
codling moth, C. pomonella. Modifications of ratios and quantities of these metabolites can change the
number of eggs laid on the plants and could modify the development of pathogens. We analyse the
soluble carbohydrate, sugar alcohol and free amino-acid composition of leaf surface water washing on
the different sites by gas chromatography. On the first set of analysis, we observed on the upper side
of the distal leaf surfaces one day after spraying that the treatment increased the quantity of saccharose
(from 180 µg/cm² on the untreated trees up to 243 µg/cm²) and sorbitol (from 89 up to 135 µg/cm²)
compared to the non treated trees. Analysis of free amino acids did not show any significant difference
on the distal leaves on both leaf sides. We are currently doing a second set of analysis using liquid
chromatography.

Key words: biocontrol agent, codling moth, epiphytic microorganisms, powdery mildew, scab,
metabolites

Introduction

Biocontrol agents are used as control method against different pathogens like Podosphaera
leucotricha (causal agent of apple powdery mildew) such as the bacterium Bacillus subtilis,
active ingredient in Serenade®. In this project, we first screened epiphytic yeasts and bacteria for their ability to decrease the severity of apple powdery mildew and their capacity to develop on the apple tree canopy. We look at the effects on the germination of *Venturia inaequalis* conidia, on the egg quantities laid by the codling moth, *Cydia pomonella* on trees, their distribution within the tree and on the chemical composition of the phylloplane, as it is known that primary metabolites (sugars) of the phylloplane stimulate the egg laying of the codling moth.

**Materials and methods**

All the experiments were conducted on apple tree ‘Golden Delicious’ from 3-4 years old cultivated in containers.

**Microorganisms screening to control apple powdery mildew**

The microorganisms were grown at 25°C in liquid media on a rotary shaker (100 rpm for 48 h); the bacteria in nutrient broth and the yeasts in YMDB. Cultures were then centrifuged at 5°C, 5000 rpm for 10 min and re-suspended in sterile water. The treatment consisted of a suspension containing c. 150 ml/tree and was sprayed at 5:00 pm solar time. Cell concentration was evaluated using a standard curve and measuring the optical density with a spectrophotometer at 450 nanometer for the yeast and 650 nm for the bacteria.

Microorganisms, four epiphytic yeast and five epiphytic bacteria strains were applied once every seven days. Disease incidence (%infected leaves) and severity (% diseased leaf area) were evaluated and AUDPC values were calculated, scoring 5 fully expended younger leaves on 5 randomly chosen branches per microorganism. Assessments were carried out 14, 21 and 28 days after the first treatment, starting with the symptom appearance.

**Microorganism preparation for the side-effect studies**

For all the following experiments yeast isolate Y16 was selected because of its effect on powdery mildew and its capacity to develop on the apple tree canopy. Before treatment, the yeast suspension was cleaned from the growth media by a double centrifugation of 20 minutes at 4°C. The cells were then suspended in water at a concentration of 10^7 cells/ml.

**Yeast effect on the conidial germination of *V. inaequalis***

Seedlings of cv. Golden Delicious with 4-5 leaves were used. The youngest opened leaf of each seedling was marked. For each set of experiments, the seedlings were divided into three homogenous groups (12-18 plants each). Seedlings were treated with i. distilled water, ii. yeast suspension, and iii. yeast supplemented with 0.01% Tween 80 and three hours later, once the leaves were dry, *V. inaequalis* conidia were inoculated on leaves following the method described by Gessler & Stumm (1984). The concentration of conidia was adjusted to c. 10^4 using a Thomas-Kammer haemocytometer. The seedlings were then placed above a tray full of water and the whole structure was covered by a polyethylene bag in order to reach a humidity level closed to 100%, in darkness at 18°C for a 48 hours infection period. At 3, 5 and 7 days after the inoculation, the marked leaves were prepared for microscope observations (Silfverberg-Dilworth, 2003). The number of non-germinated conidia and the number of germinated and penetrated was counted under a light microscope (Olympus BH-2). The percentage of germinated and of penetrated conidia between the different treatment were then compared using the student test and the program STATISTICA version 7.0.

**Yeast effect on the codling moth oviposition in semi-natural condition trials**

The experiment was conducted in the Trentino, Italy, during the second flight of the codling moth corresponding to the month of August in 2005 and repeated in 2006. This experiment was carried under a roof in order to have a light intensity close to the one in the tree canopy of
a commercial orchard. The yeast suspension was sprayed on the trees 24 hours before the insect release. Untreated trees were used as control. An experiment consisted of eight treated and eight untreated trees, (one tree = one replicate). Each tree had different tree morphology. For year 2005, the 8 replicates were separated into two groups of four replicates by a 2 day interval, with similar climatic conditions.

At 5:00 pm local time around 20 gravid females (Andermatt mass-rearing, Switzerland) were released under a white net cage, each of them containing one apple tree. Counting of eggs was made after the end of the release period on different tree sites: fruits, leaves from the corymb, from the bourse shoot and “distal” leaves (distal = all the other type of leaves). The “distal” leaves were separated into two groups at less than 20 cm from the fruits and “distal” leaves at a distance over 20 cm from the fruits, since around 95% of the eggs are laid on fruits and leaves placed less than 20 cm from fruits (Mattedi & Zelger, 2006).

**Yeast effect on the plant surface metabolites**

Samples of 6 leaves were collected in the 2005 experiment: three leaves from the corymb and three from the bourse shoot, corresponding to the preferred egg laying sites of the codling moth. Leaves from four treated trees and four untreated trees were collected and two samples of six leaves were taken from each tree at 8:00 pm solar time, during the insect release. Leaves were washed using the method described by Fiala et al. (1990). The petioles were covered with paraffin. For one sample, each side was sprayed separately with c. 10 ml of ultra-pure water per 100 cm². Sugars and sugar alcohols as well as free amino acids were quantified by liquid chromatography.

**Results and discussion**

**Microorganisms screening to control apple powdery mildew**

Only part of the results will be presented in this manuscript. Results are further analysed. The severity of the powdery mildew reaches 37.4% on the water control. It was decreases to 7.6% on the Y16 treated plants (Table 1)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H₂O</th>
<th>Y16</th>
<th>Y89</th>
<th>B16</th>
<th>B69</th>
<th>B6</th>
<th>B71</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severity (%)</td>
<td>37.4</td>
<td>7.6</td>
<td>19.8</td>
<td>9.7</td>
<td>9.7</td>
<td>10.0</td>
<td>14.1</td>
</tr>
<tr>
<td>S.E.</td>
<td>7.1</td>
<td>2.1</td>
<td>2.7</td>
<td>1.8</td>
<td>1.3</td>
<td>1.5</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Percentage of leaf area covered by symptoms, 28 days after the experiment start. Control was sprayed with water.

**Yeast effect on the conidia germination of V. inaequalis**

The yeast did not affect conidia germination and penetration by of *V. inaequalis* under the conditions of the described experiment (Fig. 1).

**Yeast effect on the codling moth oviposition in semi-natural condition trials**

In year 2005 the average quantity of eggs laid on the fruits and on leaves at less than 20 cm from the fruits (called “total prox” in the Table 2) on treated plant was more than double than on the untreated plants. On the year 2006, the average of egg quantity was 2.4 times less on the treated trees. Egg quantities laid on the control trees for the year 2005 was similar to 2006. But the yeast effect in both years is significantly different (Table 2).
Figure 1. Effect of a yeast suspension on the germination of apple scab conidia

Table 2. Raw results of the two years 2005 and 2006 of experiment

<table>
<thead>
<tr>
<th>Leaf type</th>
<th>2005</th>
<th>2006</th>
<th>2005</th>
<th>2006</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Y16+T80</td>
<td>Untreated</td>
<td>Y16+T80</td>
</tr>
<tr>
<td>Corymbe</td>
<td>6.6</td>
<td>18.9</td>
<td>5.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Distal &lt;20</td>
<td>19.2</td>
<td>31.0</td>
<td>18.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Fruits</td>
<td>6.65</td>
<td>14.0</td>
<td>6.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Borse shoot</td>
<td>7.9</td>
<td>31.7</td>
<td>7.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Total prox</td>
<td>40.4</td>
<td>95.6</td>
<td>42.4</td>
<td>17.7</td>
</tr>
</tbody>
</table>

Average of egg number laid per tree and within the tree on 8 replicates.
“Total prox” is the total of eggs laid on the fruits and on leaves at less than 20 cm from the fruits.

Yeast effect on the plant surface metabolites
The data are currently analysed and the results will not be presented here. The first analyses of the data of year 2005 tend to a general decrease of almost all substances analysed: sugar, sugar alcohols and free amino acid in the yeast treated plant.

Discussion

Among nine epiphytic microorganisms, yeast and bacterium isolates, the yeast 16 showed good potential in apple powdery mildew suppression and well develop on the apple tree canopy. However, it did not affect the conidial germination and penetration of *V. inaequalis* when applied 3 hours after pathogen infection. Nevertheless, it affected egg laying by the codling moth. Moreover, it modified the chemical composition of the phylloplane. The modification of leaf surface signals could partly explain the effect of the yeast suspension on the egg laying stimulation of the insect as already was shown in other researches (Lombarkia, 2002; Derridj & Borges, 2004). The effect of the yeast population is linked to the year. We may hypothesize that the plant–yeast interaction is linked to environmental factors, such as climatic factor.

This yeast is a potential BCA of apple powdery mildew. Before developing this yeast as a BCA, there is a need to establish the effect of this BCA on the insect since the codling moth
is economically one of the most important pest of apple trees in Europe. Although the effect of the yeast treatment is year linked, an increase of the insect damages on the production due to the BCA spray can nullify its beneficial effect gained by the control of powdery mildew.

Acknowledgement

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References

A mechanism for growth inhibition in plants, associated with *Trichoderma* application

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**Abstract:** *Trichoderma* spp. are well known for their abilities to control plant pathogens and in many cases, to enhance plant growth. Less documented is the occasional, temporary growth stunting of plants associated with application of *Trichoderma* at high doses. Trials investigating the interaction of root zone temperature and *Trichoderma* applications revealed that under conditions of high ammonium-N and high temperatures, the growth stunting effects of *Trichoderma* were increased. The symptoms shown by the trial plants resembled ammonium toxicity. These observations prompted research into the interactions between *Trichoderma* and nitrogen cycling. It was concluded that, when *Trichoderma* is added to plant root zones at high doses, and in the presence of ammonium ions, it interferes with the normal process of nitrification. This occurs either through the competitive exclusion of nitrifying bacteria in the root zone, and/or through enhanced ammonium uptake facilitated by *Trichoderma*, in a mycorrhizal type of association. As a result, the risk of ammonia toxicity and associated growth inhibition is increased. Other side effects of the combination of high levels of both *Trichoderma* and ammonium nitrogen were enhanced medium acidification, and reduced K⁺ and Ca²⁺ in leaf tissue. Even in the absence of plants, *Trichoderma* applications to soils resulted in changes in the ammonium to nitrate ratios. The levels of ammonium-N in the soil were inversely related to population size of nitrifying bacteria. This suggests that the primary mechanism of growth stunting by *Trichoderma* involves the reduction of the population of nitrifying bacteria, which in turn results in a reduced conversion of ammonium-N to nitrate-N, resulting in an increased risk of ammonium toxicity, leading to stunting.

**Key words:** ammonium toxicity, nitrogen cycle, plant growth, stunting

**Introduction**

Earlier trials conducted by Neumann and Laing (unpublished) demonstrated that when *Trichoderma* (strain Eco-T⁴) was applied in high doses to lettuce plants receiving high levels of nitrogen fertilizer as ammonium nitrogen (NH₄-N), then these plants consistently showed signs of growth inhibition and yellowing. Symptom development resembled that of ammonium toxicity. Other factors that compounded symptom development included high water temperatures and the use of comparatively sterile growing media (e.g., perlite).

Kafkafi (2001) pointed out that nitrogen, as ammonium (NH₄-N), can be beneficial when root zone temperatures are low, but detrimental when they are high. This is because ammonium is completely metabolised in the roots while nitrate is only partly reduced in the roots, with the larger part of their metabolism taking place in the leaves. With increasing root temperatures, respiration rates increase, consuming sugars. At high temperatures, no sugar is available for the metabolism of NH₄-N in the root, resulting in ammonium toxicity. This
explains the association between NH$_4$-N and water temperature but not why the application of *Trichoderma* (especially in sterile media) increased symptom development.

Further trials in experimental hydroponic systems (Neumann & Laing, unpublished) showed that the addition of *Trichoderma* at high levels (1×10$^6$ conidia/ml and above) resulted in a short-term, yet significant increase in the levels of NH$_4$-N in the re-circulating nutrient solution. This was accompanied by an increase in NH$_4$-N levels in root tissue and was inversely related to root and shoot growth. Other associated effects included root zone acidification as well as a decrease in Ca and K uptake, and an increase in Na, Fe, P, Cu and Mn in shoots. The decrease in leaf K$^+$ and Ca$^+$ provides further proof of ammonium toxicity related stresses. Haynes (1986) explained that the increased uptake of NH$_4^+$ results in the reduced uptake of cations such as K$^+$ and Ca$^+$ due to ionic competition, either with NH$_4^+$ ions *per se* or with H$^+$ ions excreted during active NH$_4^+$ uptake. The increase in Cu$^+$, Mn$^+$, Fe$^+$, Na$^+$ and P$^+$ can also be explained in terms of increased NH$_4^+$ concentration. Jeong & Lee (1996) recorded similar increases of Cu$^+$, Mn$^+$, Fe$^+$ and P$^+$ in shoot tissue of ageratum and salvia plants, with increasing NH$_4$-N levels. A possible explanation of this phenomenon is linked to the acidification effect because all the above elements are more soluble at lower pH levels.

The challenge was then to show how *Trichoderma* influences the availability and/or uptake of NH$_4$-N. Verstraete (1981) noted that, given mycorrhizae have a preference for nitrogen in the NH$_4$-N form, and therefore they could play a regulatory role as biocontrol agents of nitrification. Kleifeld & Chet (1992) showed that *Trichoderma* may function as a mycorrhizal organism and simple petri dish trials clearly showed that *Trichoderma* has a preference for NH$_4$-N (Neumann & Laing, unpublished). It was thus hypothesised that *Trichoderma* may act as a biocontrol agent of nitrification, inhibiting conversion of NH$_4$-N to NO$_3$-N, resulting in an accumulation of NH$_4$-N and therefore, creating an increased risk of ammonium toxicity. The following data goes some way towards proving this hypothesis.

**Materials and methods**

One litre plant pots were filled with growing medium (nine with composted pine bark and nine with perlite) and placed in 2l ice-cream tubs. Each pot was watered with a solution containing 500 ml water, 5 ml Voermolas (an animal energy supplement based on molasses and used to stimulate microorganism growth) and 0.5g Ocean Agriculture 3:1:3 (38) complete fertilizer (approx. 51:49% NH$_4$-N: NO$_3$-N). Prior to watering, solutions were amended with either 0.5×10$^5$ or 2×10$^6$ conidia/ml of *Trichoderma*. Each treatment was replicated three times with each medium.

Pots were left to stand for 4d, after which growing medium samples were analysed for NH$_4$-N and NO$_3$-N concentrations as well as pH. For determining growing medium NH$_4$-N and NO$_3$-N levels a Jenway Aquanova 6300 spectrophotometer was used, together with the relevant colorimetric test kits. Ten grams of medium was added to 100 ml of distilled water. The flasks were sealed with parafilm and placed on a horizontal shaker at 25°C for 30min. Samples were then filtered through a 45µm filter before analysing.

Total bacteria and nitrifying bacteria populations were determined by plate counts, using tryptone soy agar or a medium selective for nitrifying bacteria (Ball, 1997) respectively. Plates were incubated for 6d at 25°C after which CFU values were determined.

**Results and discussion**

In the pinebark medium, the initial level of NO$_3$-N was 460 µg g$^{-1}$ of medium. After 4d the level of NO$_3$-N had increased in the untreated pots to 490 µg g$^{-1}$, while decreasing to 310 µg
g⁻¹ in the pots receiving $2 \times 10^6$ *Trichoderma* conidia ml⁻¹ (four times the recommended rate for Eco-T®). NH₄-N levels did not change significantly between treatments and were measured at approximately 25 µg g⁻¹, with initial levels of 60 µg g⁻¹. This means that the percentage of NH₄-N had increased from 4.9 to 7.5% after the addition of *Trichoderma*. In the perlite medium the percentage NH₄-N increased from 20.8% (without *Trichoderma*) to 36% (four times *Trichoderma* dose). These results are summarised in Table 1 and Fig.1.

Table 1. Effects of *Trichoderma* dose on nitrogen form in different media

<table>
<thead>
<tr>
<th>Medium</th>
<th><em>Trichoderma</em> (Conidia ml⁻¹)</th>
<th>NH₄-N (µg g⁻¹ soil)</th>
<th>NO₃-N (µg g⁻¹ soil)</th>
<th>NH₄-N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pine bark</td>
<td>0</td>
<td>25 a</td>
<td>490 a</td>
<td>4.9 b</td>
</tr>
<tr>
<td>Pine bark</td>
<td>$5 \times 10^5$</td>
<td>24 a</td>
<td>380 b</td>
<td>5.9 ab</td>
</tr>
<tr>
<td>Pine bark</td>
<td>$2 \times 10^6$</td>
<td>25 a</td>
<td>310 c</td>
<td>7.5 a</td>
</tr>
<tr>
<td>Perlite</td>
<td>0</td>
<td>42 a</td>
<td>160 a</td>
<td>20.8 c</td>
</tr>
<tr>
<td>Perlite</td>
<td>$5 \times 10^5$</td>
<td>51 a</td>
<td>160 a</td>
<td>24.2 b</td>
</tr>
<tr>
<td>Perlite</td>
<td>$2 \times 10^6$</td>
<td>45 a</td>
<td>80 b</td>
<td>36 a</td>
</tr>
</tbody>
</table>

Treatments with the same letter do not differ significantly for the variable in question ($P \leq 0.05$).

Figure 1. Effect of *Trichoderma* application rate on % ammonium nitrogen relative to population size of nitrifying bacteria
The total bacterial populations in perlite media without the addition of *Trichoderma* averaged 6.3×10^{10} CFU’s on tryptone soy agar at 25°C, while the addition of *Trichoderma* at 2×10^6 spores ml^{-1} resulted in a reduction of total bacteria to 2.1×10^8 CFU’s. The number of CFU’s on nitrifying bacteria agar (Ball, 1997) also decreased from 3.3×10^8 to 4.5×10^6 with the addition of *Trichoderma*. Similar trends were observed in the pine bark medium, although overall bacterial populations were much higher. Results are shown in Table 2 and Fig. 1.

**Table 2. The effect of *Trichoderma* on bacterial population numbers in different media**

<table>
<thead>
<tr>
<th>Medium</th>
<th><em>Trichoderma</em> (spores ml^{-1})</th>
<th>Bacterial CFU’s in 10 g growing medium</th>
<th>Nitrifying bacteria (CFU’s in 10 g growing medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perlite</td>
<td>0</td>
<td>6.3×10^{10}</td>
<td>3.3×10^8</td>
</tr>
<tr>
<td>Perlite</td>
<td>5×10^5</td>
<td>2.3×10^{10}</td>
<td>8.5×10^7</td>
</tr>
<tr>
<td>Perlite</td>
<td>2×10^6</td>
<td>2.1×10^{10}</td>
<td>4.5×10^6</td>
</tr>
<tr>
<td>Pine bark</td>
<td>2×10^6</td>
<td>1.5×10^{11}</td>
<td>3.7×10^9</td>
</tr>
<tr>
<td>Pine bark</td>
<td>5×10^5</td>
<td>3.3×10^{10}</td>
<td>4.3×10^9</td>
</tr>
<tr>
<td>Pine bark</td>
<td>2×10^6</td>
<td>5.7×10^9</td>
<td>6.5×10^8</td>
</tr>
</tbody>
</table>

From the above data, the accumulation of NH_4-N was directly related to the suppression of the populations of nitrifying bacteria. *Trichoderma*, when added at artificially high levels, is capable of temporarily out-competing these bacteria, resulting in the reduced conversion of NH_4-N to NO_3-N. This in turn, increases the availability and uptake of NH_4-N by plants, with the associated increased risks of ammonium toxicity at higher temperatures. The effects are most noticeable in sterile media (e.g., perlite) due to their low biological buffering, where *Trichoderma* can establish with little microbial competition.

**References**

Ball, A.S. 1997: Bacterial Cell Culture. John Wiley and Sons Ltd., UK.
Effect of chemical pesticides and biocontrol agents on growth and mineral composition of healthy strawberries

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Abstract: Several biocontrol agents (BCAs) have shown positive effects on plant growth. It is known that triazole fungicides have an effect on plant growth, chloroplast pigments and sterol biosynthesis of maize and a "greening effect" was observed on cereals after azoxystrobin treatments. The aim of this work was to compare the effect of a triazole (penconazole), a strobilurin (azoxystrobin) and two BCAs, Trichoderma harzianum T39 and Ampelomyces quisqualis sprays, on plant growth, chlorophyll content and mineral composition of healthy strawberry plants. Plants (cv. Elasanta) were weekly treated for three times under controlled conditions, starting from the third day after planting. The total chlorophyll content was not affected by any agent. T. harzianum T39 induced a temporary increase (not observed with the other treatments) of plant growth in the first two weeks post treatment, compared to untreated control, but at the end of the experiment the total leaf surface of T. harzianum T39 treated plants was similar to the other treated and untreated plants. Azoxystrobin and penconazole induced an increase in the development of aerial parts, which seems to be associated with a decrease in root growth. Azoxystrobin treatments increased phosphorus content in stem and leaves, while penconazole reduced potassium in leaves and other microelements in leaves and roots. T. harzianum T39, penconazole and azoxystrobin seem to affect the physiology of strawberry plants, conversely A. quisqualis, which is known to act as a powdery mildew hyperparasite, seems not to interfere with it.

Key words: Ampelomyces quisqualis, Azoxystrobin, penconazole, Trichoderma harzianum T39

Introduction

Several bacterial and fungal biocontrol agents have shown positive effects on plant growth (Camprubi et al, 1995; Kleifeld & Chet, 1992; Srinath et al., 2003). Triazole fungicides have an effect on growth, chloroplast pigments and sterol biosynthesis of maize (Khalil et al., 1990). A "greening effect" was observed on cereals after azoxystrobin treatments (Bertelsen et al., 2001). Under commercial growing conditions strawberry plants treated with fungicides or biocontrol agents apparently look bigger compared to sulphur or untreated plants. In the field it is difficult to separate the indirect effect of pathogen control obtained with treatment from a possible direct effect on plant physiology. The aim of this work was to detect a potential effect of a triazole (penconazole), a strobilurin (azoxystrobin) and two biocontrol agents, Trichoderma harzianum T39 and Ampelomyces quisqualis, on plant growth, chlorophyll content and mineral composition of healthy strawberry plants.
Material and methods

Trials were performed in a pathogen-free greenhouse in S. Michele all’Adige (Italy) on the most widely used soil-less strawberry (*Fragaria x ananassa* Duchense) cultivar Elsanta. During the experiments, the temperature was 22±3°C and relative humidity was 65±15%. The experiment was arranged in completely randomised blocks using four pots (replicates) having six plants each per treatment. The trial was repeated twice. Treatments (Table 1) were weekly applied (three times), starting from the 3rd day after planting.

Table 1. Active ingredient and name of the commercial product, percentage of each active ingredient in the commercial product and dosages used in the experiment

<table>
<thead>
<tr>
<th>Active ingredient (a.i)</th>
<th>Commercial product</th>
<th>a.i. (%)</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoxystrobin</td>
<td>Ortiva</td>
<td>23.2</td>
<td>0.8 ml/l</td>
</tr>
<tr>
<td>Penconazole</td>
<td>Topas</td>
<td>10.5</td>
<td>0.4 ml/l</td>
</tr>
<tr>
<td>Sulphur</td>
<td>Thiovit</td>
<td>80</td>
<td>3.0 g/l</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em> T39</td>
<td>Trichodex</td>
<td>6</td>
<td>4.0 g/l</td>
</tr>
<tr>
<td><em>Ampelomyces quisqualis</em></td>
<td>AQ10</td>
<td>58</td>
<td>0.08 g/l</td>
</tr>
<tr>
<td>Water</td>
<td>Untreated control</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Assessments for the estimation of growth rate were carried out on 3 random sampled plants/replicate by counting the total number of leaves (twice a week) and measuring the leaf area (weekly). Wet and dry weight of leaf, petiole and root of each plant were assessed at the end of experiment (27 days after planting). The chlorophyll content was determined with a SPAD chlorophyll meter (Minolta) and the mineral composition of leaves, petioles and roots was analysed with standard GLP tests for leaf mineral composition of strawberry (IASMA laboratories). Area under “number of leaves present” curve (AUNLP) during the experiment was calculated according to the following formula with $t_b$ and $t_e$ respectively beginning and end of the experiment, $t$ is the time in days and $y$ is the total number of leaves/plant.

\[
\int_{t_b}^{t_e} \frac{y}{dt}
\]

Statistical analyses (ANOVA) of the data were performed using the Statistica software 6.0 (Statsoft) after suitable transformations to normalize data. Means were separated using Fisher’s test at $P<0.05$.

Results and discussion

In all treatments bloom began at the same time (14 days after planting) and emission of new leaves stopped 21 days after planting. SPAD varies between 48 and 50 units, with no significant difference among treatments. Sulphur shower a chlorophyll content of 43 units, significantly lower than the values of the other treatments, effect mainly due to its phytotoxicity. AUNLP calculated during the experiments was similar among treatments (Table 2). Plants treated with *T. harzianum* T39 grew faster after the first treatment (Fig. 1) compared to all treatments (significant difference at one week), that was nullified at the end of the growing cycle. *T. harzianum* T39 treated plants had smaller leaves compared to plants treated with azoxystrobin (Table 2). Treatments did not influence the petioles weight; azoxystrobin and penconazole had heavier leaves and lighter roots compared to the untreated (Table 3). Similar results were obtained on dry and wet weight of root, petiole and leaf.
Azoxystrobin treatments increased four times phosphorus content of leaves and decreases K, Mg, S, Mn and Bo in roots, while penconazole reduced potassium in leaves (-12%) and other macro and microelements in leaves and roots (Ca, Mg, S, Mn, Bo and Cu) (data not shown). *T. harzianum* T39 applied on leaves did not influence mineral composition of leaf and root, differently from what was found by Yedidia et al., 2001, who showed that *T. harzianum* T-203, applied on roots, changes mineral composition of plants.

Table 2. Number of leaves in the second assessment, area under leaf growth curve (AUNLP) and the average leaf area/plant at the end of the experiment

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>N. leaves/plant*</th>
<th>AUNLP**</th>
<th>Leaf area (cm²/plant)***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoxystrobin</td>
<td>15.8 b</td>
<td>334 a</td>
<td>229.5 a</td>
</tr>
<tr>
<td>Penconazole</td>
<td>15.6 b</td>
<td>349 a</td>
<td>208.4 ab</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em> T39</td>
<td>18.5 a</td>
<td>397 a</td>
<td>164.5 ab</td>
</tr>
<tr>
<td><em>Ampelomyces quisqualis</em></td>
<td>16.2 b</td>
<td>346 a</td>
<td>215.5 ab</td>
</tr>
<tr>
<td>Water</td>
<td>16.6 b</td>
<td>349 a</td>
<td>217.2 ab</td>
</tr>
</tbody>
</table>

* Second assessment (after 7 days).

** Area under “number of leaves present” curve (AUNLP) during the experiment, according to the following formula with *t₀* and *tₑ* are respectively beginning and end of the experiment, *t* is the time in days and *y* is the total number of leaves/plant.

*** At the end of experiment (27 days after planting).

Numbers in each column followed by different letters are significant different according Fisher’s test at *P*<0.05.

In conclusion azoxystrobin is not producing a “greening effect” on strawberry as seen on other crops, in fact total chlorophyll content did not vary among the treatments. *T. harzianum* T39 induced a temporary increase (not observed with the other treatments) of plant growth in the first two weeks post treatment, but at the end of the experiment the total leaf
surface of *T. harzianum* T39 treated plants was similar to the untreated plants. Azoxystrobin and penconazole treatments induce an increase in the development of aerial parts (leaf weight), which seems to be associated with a decrease in root growth. *T. harzianum* T39, penconazole and azoxystrobin seems to affect the physiology of strawberry plants, either plant growth or mineral composition, conversely, *A. quisqualis*, which is known to act as a powdery mildew hyperparasite, seems not to interfere with it.

Table 3. Leaf and root wet weight at the end of the experiment (27 days after planting). Treatments were applied weekly (three times)

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Leaf wet weight (g/plant)</th>
<th>Root wet weight (g/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoxystrobin</td>
<td>46.9 a</td>
<td>154.7 b</td>
</tr>
<tr>
<td>Penconazole</td>
<td>41.1 ab</td>
<td>139.7 b</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em> T39</td>
<td>37.0 bc</td>
<td>172.6 a</td>
</tr>
<tr>
<td><em>Ampelomyces quisqualis</em></td>
<td>32.5 c</td>
<td>168.2 a</td>
</tr>
<tr>
<td>Water</td>
<td>31.4 c</td>
<td>172.3 a</td>
</tr>
</tbody>
</table>

Numbers in each column followed by different letters are significant different according Fisher's test at $P \leq 0.05$.

Acknowledgements

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References


Occurrence of bacteriophages against antagonists towards *Verticillium dahliae* Kleb. in the rhizosphere of strawberry

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**Abstract:** Much research has been done on antifungal bacteria naturally occurring in the rhizosphere of crops, because they get more attention referring to control of soilborne fungal diseases in an environmentally friendly way. Many factors can affect the persistence and activity of these beneficial bacteria. The occurrence of bacteriophages can reduce the abundance of introduced bacteria and consequently their disease-suppressing effect. New antagonistic bacteria towards the plant pathogenic fungus *Verticillium dahliae* Kleb. were isolated from the rhizospheres of strawberry. The proportion of antagonistic bacteria ranged from 5.9 to 14.8%. Phages were isolated from the same samples as the bacterial antagonists by using these bacteria as hosts. In strawberry rhizosphere the proportion of phage-sensitive antagonists ranged from 40.0 to 62.5%, depending on the sampling time. The highest number of ten phage-host-systems per sample was isolated from young plants representing an abundance of culturable phages of about $1.2 \times 10^4$ PFU (plaque forming units) g$^{-1}$ rhizosphere sample. The counts of viral particles were estimated as up to $1 \times 10^9$ particles g$^{-1}$. All phage-sensitive antagonists were identified as similar to each other (>70% by BOX-PCR fingerprint pattern) and to *Pseudomonas fluorescence* (sequencing of the 16S rRNA gene). The phages formed plaques with different morphology, but all phages lysed all isolated host strains. By RFLP 10 different phage groups could be determined. The phages are to be characterized by transmission electron microscopy.

**Key words:** *Verticillium*-antagonists

**Introduction**

Bacteriophages are ubiquitous in nature and occur in densities of $2.5 \times 10^8$ ml$^{-1}$ in aquatic habitats (Bergh et al., 1989, Hennes & Simon 1995) and about $1.5 \times 10^7$ g$^{-1}$ in soil (Ashelford et al., 2003). It has been suggested that they are important in both controlling bacterial numbers and in facilitating bacterial gene transfer (Proctor & Fuhrman, 1990, Kokjohn & Miller, 1992). Phages are mostly present in environments with high densities of metabolic active bacteria as potentially host strains. Such an environment is the plant rhizosphere. Much research has been done in isolating, studying and using rhizosphere bacteria as natural antagonists towards fungal phytopathogens (Weller, 1988; Berg et al., 2001; Scherwinski et al., 2006). Only one study exists, dealing with the potential impact of bacteriophages on survival and biocontrol activity of an effective biological control agent (Keel et al., 2002).

The present study investigates the occurrence of bacteriophages of antagonists to *Verticillium dahliae* Kleb. in the rhizosphere of strawberry within three sampling times. The new isolated phage-host-systems were characterized in order to find an efficient model system for further studies. Additionally, the viral abundances of the samples were estimated by epifluorescence microscopy.
Material and methods

Planting and sampling
Young strawberry plants (cultivar Elsanta) were planted after cleaning the roots three times in water and once in sterile distilled water to minimize the influence of soil bacteria and phages. Prior planting, two and four weeks after planting, rhizosphere samples were taken. A sample consisted of 10 g roots with adhering soil which were combined from three plants each.

Isolation of bacteria
The samples were treated and plated onto R2A agar (Difco, Detroit, USA) according to the method of Berg et al. (2002).

Screening of antagonistic bacteria
Bacterial isolates were screened for their activity towards *Verticillum dahliae* Kleb. V25 by dual-culture in vitro assay on Waksman agar according to the method of Berg (1996). The formation of an inhibition zone of fungal growth around the tested isolate indicated an antagonistic activity of the isolate.

Isolation of phages and determination of phage-sensitive antagonists
The antagonistic bacteria towards *V. dahliae* were used as host strains to isolate phages from the same sample by the soft-agar-technique (Adams 1959). The phage fraction of the sample was obtained by two centrifugation steps: (i) 5 min 450×g to remove large particles and (ii) 20 min 4500×g to remove bacteria. The supernatant was filtered through 0.2 µm sterile filter. One ml of the sterile filtered sample (phage fraction) was mixed together with 1 ml of a 1h-growing bacterial culture (in nutrient broth). Five ml of soft agar (0.6%, 50°C) were added to the mixture and flowed over the agar in a Petri dish. The occurrence of phages was shown after 24 h by the formation of single plaques in the bacterial lawn. The proportion of “phage-sensitive antagonists” was determined. Phages were isolated by a plaque isolation in SM phage buffer (Sambrook & Russell, 2001). The isolation technique was repeated three times successively. To produce high concentrated phage stock solutions plates with confluent lysis were prepared. The phages were stored in SM phage buffer at 4°C.

Determination of the phage numbers
Using the phage-sensitive antagonists as host strains the Plaque Forming Units (PFU) of the samples were determined by the soft-agar-technique. The phage fraction of the sample was diluted in SM phage buffer.

Characterization and identification of the phage-sensitive antagonists
The phage-sensitive *Verticillum* antagonists were characterized by their BOX-PCR fingerprint pattern according to the method of Berg et al. (2002). Finally the BOX-PCR fingerprints were clustered using GelCompar® software (version 4.1, Applied Math, Kortrijk, Belgium). Some bacteria were selected for identification by partly sequencing of 16S rRNA gene (about first 700 bp) followed by an alignment with reference sequences using the BLAST algorithm (Altschul et al., 1997). The 16S rRNA gene PCR product was sequenced directly using the 27F eubacterial primer (Lane, 1991) by GATC Biotech AG (Konstanz).

Characterization of the phages
The phages were characterized by their plaque morphology (size, turbidity, with or without a halo), host range and restriction pattern with the enzyme BstEII. The Phage DNA was isolated according to the method of Sambrook & Russell (2001). The restriction was prepared according to manufacturer’s manual for 2 h at 37°C. The agarose gel electrophoresis was carried out in 0.8% agarose and 1×TAE for 3 h at 110 V.
**Viral counts by epifluorescence microscopy using SYBR Gold**

SYBR Gold (Molecular Probes, Inc., Eugene, OR, USA) was diluted 1:10 with 0.02 µm filtered sterile deionized water and additionally 1:1000 in 0.1 to 1 ml of the phage fraction of the sample. The sample was incubated in dark for about 15 min and filtered through a 0.02 µm pore size Al₂O₃ Anodisc 25 membrane filter (Whatman), backed by a 0.45 µm cellulose mixed ester membrane (Millipore). The filter was placed on a glass slide with a drop of immersion oil under and above the filter and above the cover slip. Slides were counted immediately on an Olympus BX51 epifluorescence microscope with 100× objective under blue excitation.

**Results and discussion**

**Proportion of antagonistic isolates and phage-sensitive antagonists**

The proportion of antagonistic isolates in the strawberry rhizosphere ranged from 5.9 to 14.8%, with respect to the sampling time (Table 1). The proportion of phage-sensitive antagonists was determined between 40 and 62.5% with the highest numbers on young strawberry plants. This is a first insight and further studies have to be done.

**Phage numbers**

The high proportion of phage-sensitive antagonists resulted in 10, 6 and two new phage-host-systems (PHS) (Table 1). Using these, phage numbers of up to 6.2 ×10⁵ PFU g⁻¹ rhizosphere sample were obtained. The phage numbers did not correlate with the numbers of PHS. Ashelford et al. (1999) reported a peak density of culturable phages of about 10⁵ PFU g⁻¹ on the surface of field-grown sugar beet.

**Table 1. Characterisation of antagonists and phages in the rhizosphere of strawberry at three sampling times**

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Proportion of antagonists (%)</th>
<th>Proportion of phage-sensitive antagonists (%)</th>
<th>Number of isolated PHS*</th>
<th>Phage numbers (PFU g⁻¹)</th>
<th>Viral numbers (Particles g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior planting</td>
<td>14.81</td>
<td>62.5</td>
<td>10</td>
<td>1.2 ×10⁴</td>
<td>2.3 ×10⁸</td>
</tr>
<tr>
<td>Two weeks</td>
<td>11.76</td>
<td>54.5</td>
<td>6</td>
<td>6.2 ×10⁵</td>
<td>9.6 ×10⁹</td>
</tr>
<tr>
<td>Four weeks</td>
<td>5.88</td>
<td>40.0</td>
<td>2</td>
<td>2.1 ×10⁴</td>
<td>3.1 ×10⁹</td>
</tr>
</tbody>
</table>

*PHS: phage-host-system

**Characterization and identification of phage-sensitive antagonists**

All of the 18 phage-sensitive antagonistic isolates showed very similar BOX-PCR patterns of about 70% similarity. Eight strains were identified as related to *Pseudomonas fluorescens* by sequencing a part of the 16S rRNA gene. The majority of so far identified antagonistic strains in the rhizosphere of strawberry belonged to the pseudomonads (Berg et al., 2003). Pseudomonads show a high rhizosphere competence and metabolic activity. These features could make them suitable as potential phage host strains.

**Characterization of the phages**

According to their plaque morphology up to three phages were isolated of each phage-sensitive antagonist. All of the 44 phage isolates lysed all phage-sensitive antagonists. This was expected because of the high similarity of the host strains. Molecular profiling (RFLP) of the phages resulted in 10 different phage-groups. One group consisted of 2 to 9 phage
isolates. Mostly, the phages of one host were identically, the two or three phage isolates of each of 12 hosts showed the same pattern. Two different phages were isolated for each of three host strains. For all of 5, 3 and 2×2 strains phages with an identical pattern were obtained, independently from the sampling time. Phages with similar plaques showed different molecular profiles. The plaque morphology should not be considered as discriminating feature, but is a first hint to different phages. In a further step, the phages should be characterized by transmission electron microscopy.

**Viral counts by epifluorescence microscopy**

By epifluorescence microscopy viral numbers of up to 9.6×10⁹ particles g⁻¹ were counted in the strawberry rhizosphere (Table 1). The highest number was obtained after two weeks. Other detected numbers of virus particles in soil ranged at about 1.5×10⁷ g⁻¹ by transmission electron microscopy (Ashelford et al., 2003). The discrepancy between the high viral numbers and the numbers of culturable phages in the present study reflect the high diversity of virus particles in soil, which can be culturable phages of other bacteria, unculturable phages, small virus particles of algae or other prokaryotes. The present study is a first approach. Further studies about the possible impact of phages on the bacterial host population considering the potential biocontrol activity of the host strains should follow.

**Acknowledgements**

The author thanks Prof. Mardjan Arvant for the help with GelCompare software, PD Dr. Rhena Schumann to use the epifluorescence microscopy equipment and the students Susanne Helm and Leona Trübe for their technical assistance. The study is supported by the Deutsche Forschungsgemeinschaft.

**References**


Wound age effect on the efficacy of *Candida oleophila* strain O against post-harvest decay of apple fruits

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**Abstract:** In the present work, wound age effect on the efficacy of *Candida oleophila* strain O against *Botrytis cinerea* was assessed on harvested apple fruits. To this end, a *C. oleophila* strain O suspension was applied to apple wounds 0, 30, 60, 120, 180 or 240 min. after wounding. One hour after treatment, wounds were inoculated with a conidial suspension of *B. cinerea* and the population of the antagonist at the wound site was recovered and quantified. After an incubation period of 7 d, the lesion diameter caused by *B. cinerea* was measured and the protective level of strain O estimated. There was no effect of the tested periods on the population size of strain O one hour after its application on apple wounds (i.e., moment of *B. cinerea* inoculation). In the absence of the antagonistic strain, the extent of the lesions depends on the freshness of the wound at the inoculation time. The lesion diameter of the infection was in fact smaller in fresh (0 and 30 min after wounding) than in old (60 to 240 min after wounding) wounds. In the presence of strain O, there was a significant reduction of the infection lesions regardless of the lag period between wounding and treatment. The protective level decreased with increasing time lapse between wounding and treatment, the highest protection being obtained when strain O was applied on fresh wounds. This suggests that *C. oleophila* strain O should be applied immediately after wounding. As postharvest decay of apples is mainly due to handling operations occurring between harvest and storage, an application of the antagonistic strain O as soon as possible after harvest is recommended for an optimal biocontrol of postharvest decay caused by *B. cinerea* on apple fruits.

**Keywords:** biological control, *Botrytis cinerea*

**Introduction**

Gray mould rot caused by *Botrytis cinerea* is one of the most devastating diseases of stored apple fruits. The yeast *Candida oleophila* strain O was isolated from the surface of Golden Delicious apples and selected for its greater effectiveness against this post-harvest pathogen (Jijakli et al., 1993).

Wounds inflicted to apple fruits are the primary site for infection by conidia of postharvest pathogens. Immediately after wounding, a massive and temporary generation of reactive oxygen species (ROS) was detected in apple wounds during the first four hours following wounding (Castoria et al., 2003). While moisture in fresh wounds is favourable for an optimal microbial growth, this parameter may rapidly be limiting as the wound surface dries (Mercier & Wilson, 1995). After wounding, apple fruits were found to exhibit healing by formation of wall thickenings (Lakshminarayana et al., 1987). These reported data suggest that the post-wounding prevailing conditions at the wound site are continually changing with increasing incubation periods.

In practical situations, the delays between wounding and biocontrol treatment may be of hours or even days. The behaviour of the antagonist agent after its application could thus
depend on the freshness (age) of the apple wound. Knowledge of the effect of this parameter on the efficacy of *C. oleophila* strain O against *B. cinerea* could help in the development of an effective biological control method against gray mould on postharvest apple fruits.

**Materials and methods**

The present work has required apple fruits (cv. Golden Delicious), a culture of the antagonist *Candida oleophila* strain O previously subcultured each day (3 times) on potato dextrose agar (PDA) at 25°C, and a 10- to 15-day-old PDA culture of the pathogen agent *Botrytis cinerea* incubated at 25°C.

Apple fruits were surface disinfected with 10% sodium hypochlorite for 2 min and rinsed with sterile water. Then, each apple was wounded 3 times, the wounds being 4 mm in diameter and about 3 to 4 mm deep. Wounds were after that treated with 40 µl of strain O suspension (10^7 cfu/ml) 0, 30, 60, 120, 180 or 240 min after wounding. For each period, the control received water only. One hour after strain O treatment, two different experiments were conducted. For the first one, a set of wounds was used for the estimation of the antagonist population (5 apples per period). Strain O cells were recovered by pipeting and then diluted-plating for a colony forming unit (CFU) assay. For the second experiment, wounds (15 apples per treatment) were inoculated with 40 µl of a conidial suspension of *B. cinerea* (10^6 conidia/ml). Fruits were stored under high humidity in enclosed plastic trays and incubated in the dark at 25°C for 7 d, after which the lesion diameter caused by *B. cinerea* infection was measured. The protective level (P) was estimated according to the formula (Dc-Da)/Dc x 100, where Dc and Da are respectively the diameter lesion of the control and treated apples.

Data were subjected to analysis of variance. Means were separated using the Duncan’s multiple range test (*P* ≤ 0.05) using the Statistical Analysis System (SAS) program.

**Table 1.** *C. oleophila* strain O population size (10^4 cfu/wound) after one hour of incubation in apple wounds

<table>
<thead>
<tr>
<th>[Wounding - Strain O application] min</th>
<th>Strain O population (10^4 cfu/wound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.38 ± 0.20a</td>
</tr>
<tr>
<td>30</td>
<td>4.16 ± 0.60a</td>
</tr>
<tr>
<td>60</td>
<td>4.43 ± 0.50a</td>
</tr>
<tr>
<td>120</td>
<td>4.60 ± 0.26a</td>
</tr>
<tr>
<td>180</td>
<td>4.81 ± 0.64a</td>
</tr>
<tr>
<td>240</td>
<td>5.56 ± 0.24a</td>
</tr>
</tbody>
</table>

Strain O (40 µl of 10^7 cfu/ml) was applied after increasing periods following wounding (0 - 240 min). Five apples with three wounds each were used per period. Each value represents mean ± standard error. Values with the same letter are not significantly different (*P* ≤ 0.05).

**Results**

Table 1 displays that, after one hour of incubation in apple wounds, the size of the recovered population of the applied strain O was not affected by the wounding to treatment interval time. Fig. 1 shows that the extent of the lesions caused by *B. cinerea* depends on the presence or the absence of strain O as well as on the freshness of the wounds at the treatment/inoculation time. In the controls (without strain O), the lesion diameter of the
infection caused by *B. cinerea* was significantly lower in fresh (0 and 30 min after wounding) than in old (60, 120 and 240 min after wounding) wounds. In the presence of strain O, there was a significant reduction of the infection lesions regardless of the lag period between wounding and treatment (Fig. 1).

The highest protective level was obtained when strain O was applied either immediately (0 min) or 30 min after wounding (Fig. 2). In case of wounds treated 60 to 240 min after wounding, the protection was reduced in comparison with freshly-made wounds. As the population size of strain O in apple wounds was the same whatever the moment *B. cinerea* was inoculated (Table 1), the reduction of the protective level in old wounds (60 – 240 min after wounding) may be ascribed to the important development of the infection in these conditions (Fig. 1).

![Figure 1](image1.png)

**Figure 1.** Lesion diameter (cm) on wounded apple fruits after an incubation period of 7 d. Each wound was first treated with 40 µl of a suspension of *C. oleophila* strain O (10^7 cfu/ml) 0, 30, 60, 120, 180 and 240 min after wounding and then inoculation with 40 µl of a *B. cinerea* suspension at 10^6 spores/ml. Each value represents mean ± standard error. Histograms with the same letter(s) are not significantly different (P ≤ 0.05).

![Figure 2](image2.png)

**Figure 2.** Protective level (%) of *C. oleophila* strain O against *B. cinerea* on wounded apple fruits after an incubation period of 7 d. Each wound was first treated with 40 µl of a suspension of *C. oleophila* strain O (10^7 cfu/ml) 0, 30, 60, 120, 180 and 240 min after wounding and then inoculation with 40 µl of a *B. cinerea* suspension at 10^6 conidia/ml.
Discussion

In our previous works dealing with the use of the yeast *C. oleophila* strain O as a potential biocontrol agent against different postharvest pathogens on apples, citrus and tropical fruits (e.g. banana), the most studied factors controlling the level of protection were the antagonist and the pathogen concentrations as well as the time elapsing between the application of the antagonist and the inoculation of the pathogen (Jijakli et al., 1993, 2004; Lahlali et al., 2004, 2005). In the present work, the effect of another important factor, which is the lag period between wounding and the application of the antagonistic strain O (*i.e.* wound age), on the control of gray mold caused by *B. cinerea* on apples was evaluated.

Wound age may have an impact on the resistance level of apple fruits against wound pathogens (Lakshminarayana et al., 1987) and on the wound water and oxidative status (Mercier & Wilson, 1995; Castoria et al., 2003). In our case, strain O development was not affected by the wound freshness (Table 1). However, lesions caused by *B. cinerea* were more important in old than in fresh wounds. The highest level of protection was obtained when strain O was applied immediately after wounding. In general, postharvest decay is mainly due to wounds resulting from handling operations during harvest of apple fruits. For this reason, it is recommended to apply the antagonistic strain O as soon as possible after harvest for an optimal biological control of gray mold disease caused by *B. cinerea* on apple fruits.

References


Competition for amino acids as a potential mechanism of 
*Aureobasidium pullulans* against post-harvest apple blue mold

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Samir El Jaafari³, M. Haïssam Jijakli¹

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**Abstract:** *Aureobasidium pullulans* (strain Ach1-1) has proved to be very effective against *Penicillium expansum* on postharvest wounded apples. In this work, the role of amino acids in its antagonistic activity was investigated. Exogenous application of amino acids into apple wounds had significantly reduced the protective level of strain Ach1-1, the extent of the reduction being dependent on the applied concentration. HPLC analysis of apple amino acids at the wound site during the first 24-hour incubation period revealed that amino acids, especially serine, glycine and glutamic acid, were more depleted in wounds containing strain Ach1-1 alone or both strain Ach1-1 and *P. expansum* than in wounds inoculated with *P. expansum* alone or untreated wounds. Individual applications of these amino acids, most particularly serine, in apple wounds significantly decreased strain Ach1-1 efficacy against *P. expansum*. It seems thus from our data that competition for amino acids may be an important mode of action of strain Ach1-1 against *P. expansum* and serine one of the most limited amino acids in this competition.

**Key words:** apple fruits, biocontrol, nutrient competition, *Penicillium expansum*

**Introduction**

*Aureobasidium pullulans* strain Ach1-1 was selected for its high biocontrol activity against *Penicillium expansum* on wounded Golden delicious apples (Achbani et al., 2005). In a previous study, competition for nutrients was found to be a main mode of action of this strain (Krimi Bencheqroun et al., 2006). Among apple compounds, amino acids were found to be more involved than sugars and vitamins in its biocontrol activity. The purpose of the present work was to focus on the role of amino acids in the biocontrol activity of strain Ach1-1 against *P. expansum* on harvested apples and to identify those which are the most limited in the mechanism of competition.

**Material and methods**

**Effect of exogenous application of amino acid mixture on the biocontrol activity of strain Ach1-1**

The efficacy of *A. pullulans* (strain Ach1-1) against *P. expansum* (strain 880) on apple (cv. Golden Delicious) wounds was assessed as previously described (Jijakli & Lepoivre, 1993) with some modifications (Krimi Bencheqroun et al., 2006). Amino acid solutions were prepared by mixing most of those known to be present in apple tissues at concentrations 2, 10 and 20 times the concentration reported for apple fruits (USDA nutrient database for standard
reference, release 14, 2001). Amino acids were applied (40 µl per wound) one hour after the pathogen inoculation. Controls were treated with the same amino acid solutions in the absence of the antagonist. Lesion diameters and the protective level were estimated according to Krimi Bencheqroun et al. (2006).

**Time-course evolution of amino acids in apple wounds**

Wounded apples were distributed into four sets: Set 1: non treated apples (control); Set 2: application of strain Ach1-1 alone (antagonist); Set 3: inoculation with *P. expansum* alone (pathogen); Set 4: application of strain Ach1-1 followed by the inoculation with *P. expansum* (antagonist + pathogen). For each set, culture solutions in wounds were extracted by pipeting 0, 4, 6, 14 and 24 hours after the application of the antagonist and their amino acid concentrations were determined by HPLC.

**Effect of individual applications of some specific amino acids on the biocontrol activity of strain Ach1-1**

Serine, glycine, glutamic acid and alanine were selected for biocontrol assays in wounds apple. The evaluation of individual effects of these amino acids on Ach1-1 efficacy was performed as above at the high concentration (20 times) only.

**Statistical analysis**

For biocontrol assays, 15 apple fruits were used per treatment (3 wounds per apple). Each test was conducted twice and data were subjected to analysis of variance. Means were separated using the student - Newman - keul’s at $P \leq 0.05$. All analyses were performed using the Statistical Analysis System (SAS/STAT) software.

**Results**

**Effect of exogenous application of amino acid mixture on the biocontrol activity of strain Ach1-1**

In controls, lesion diameters were increased with increasing amino acid concentrations (Table 1). The application of strain Ach1-1 significantly reduced these infection lesions, the extent of the reduction being dependent on the applied concentration. The higher the amino acid concentration, the lower was the protection level of strain Ach1-1 against *P. expansum*.

Table 1. Effect of exogenous amino acid application in apple wounds on lesion diameter (cm) developed by *P. expansum* strain 880 after 5 days of incubation in the absence (Control) or in the presence of *A. pullulans* strain Ach1-1 (Strain Ach1-1) and on the corresponding levels of protection (%)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesion diameter (cm)</th>
<th>Protective level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Strain Ach1-1</td>
</tr>
<tr>
<td>None</td>
<td>1.30 ± 0.04</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Amino acids (2x)</td>
<td>1.60 ± 0.03</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>Amino acids (10x)</td>
<td>1.87 ± 0.02</td>
<td>0.80 ± 0.03</td>
</tr>
<tr>
<td>Amino acids (20x)</td>
<td>2.00 ± 0.02</td>
<td>1.34 ± 0.03</td>
</tr>
</tbody>
</table>

Values with the same letter are not significantly different ($P \leq 0.05$).

**Time-course evolution of amino acids in apple wounds**

Fig. 1 shows that the concentration of amino acids in apple wounds decreased during apple incubation. The most important decrease was observed in apple wounds treated with strain
Ach1-1 and especially in those containing both strain Ach1-1 and *P. expansum*. The detailed analysis of the different amino acids has shown that serine, glycine and glutamic acid were amino acids whose concentration was largely reduced with incubation time comparatively to the other amino acids (e.g. alanine) (detailed data not presented).

**Effect of individual applications of some specific amino acids on the biocontrol activity of strain Ach1-1**

Lesion diameters of the controls were not significantly affected by the exogenous application of individual amino acids (except in the case of glutamic acid) (Table 2). Strain Ach1-1 significantly reduced lesions developed by *P. expansum* regardless of the amino acid identity. The lowest level of protection was obtained with serine indicating that this amino acid may be the most involved in competition.

![Figure 1. Amino acid concentration (nmol/ml) in apple wounds during the first 24-hour incubation period. Wounds were either non treated (control), treated with strain Ach1-1 alone (antagonist), inoculated with *P. expansum* alone (pathogen) or treated with strain Ach1-1 and then inoculated with *P. expansum* (antagonist + pathogen).](image)

Table 2. Effect of adding specific amino acids in apple wounds on lesion diameter (cm) developed by *P. expansum* strain 880 after 5 days of incubation in the absence (Control) or in the presence of *A. pullulans* strain Ach1-1 (Strain Ach1-1) and on the corresponding levels of protection (%)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Lesion diameter (cm)</th>
<th>Protective level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Strain Ach1-1</td>
</tr>
<tr>
<td>None</td>
<td>2.25 ± 0.03 a</td>
<td>0.28 ± 0.07 f</td>
</tr>
<tr>
<td>Serine</td>
<td>2.15 ± 0.05 a</td>
<td>1.45 ± 0.06 c</td>
</tr>
<tr>
<td>Glycine</td>
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<td>1.19 ± 0.10 d</td>
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<tr>
<td>Glutamic acid</td>
<td>1.87 ± 0.00 b</td>
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<tr>
<td>Alanine</td>
<td>2.07 ± 0.00 a</td>
<td>0.74 ± 0.10 e</td>
</tr>
</tbody>
</table>

Values with the same letter are not significantly different (*P*≤0.05).

**Discussion**

Recently, we provided *in vitro* and *in situ* evidence that the biocontrol activity of strain Ach1-1 against *P. expansum* essentially relies on competition for nutrients, especially for amino
acids (Krimi Bencheqroun et al., 2006). In this study, we showed that an exogenous application of increasing concentrations of amino acids in apple wounds significantly lowered the biocontrol activity of strain Ach1-1 against *P. expansum* (Table 1) without altering the development of these microorganisms. At the highest concentration (20×), the protective level was reduced by about 64% (32.8 vs. 91%), suggesting once again that competition for apple amino acids by strain Ach1-1 plays an important role in suppressing *P. expansum*. This result was confirmed by a time-course analysis of wound amino acids during apple incubation which revealed that these amino acids were more depleted in the presence of strain Ach1-1 than in the presence of *P. expansum* and that the most rapid depletion was obtained in the presence of both agents (Fig. 1). In a previous work, *A. pullulans* was able to assimilate three major amino acids (aspartic acid, serine, and glutamic acid) present in apple juice during 24 hours of incubation (Janisiewicz et al., 2000). In our case, two of these amino acids (serine and glutamic acid) as well as glycine were amino acids whose concentration was the most affected during incubation (data not shown). Based on biocontrol assays, serine seems to be the most limited amino acids as its application strongly lowered the biocontrol activity of strain Ach1-1.

As a conclusion, our data provide strong evidence that competition for apple amino acids would be one of the main mechanisms of action involved in the biocontrol activity of the antagonist *A. pullulans* strain Ach1-1 against *P. expansum* on postharvest apples. Among these amino acids, serine appears to be the most limited nutrient in this competition. The investigation will continue to find out the gene(s) involved in the uptake and the metabolisation of serine by the antagonist cells.

Acknowledgements

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In vitro study of the influence of temperature, pH, and $a_w$ on the growth rate of *Trichoderma asperellum*

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Abstract: The effects of water activity ($a_w$), temperature and pH were evaluated on the radial growth of *Trichoderma asperellum* (strains PR10, PR11, PR12, 659-7), an antagonist of *Phytophthora megakarya*, the causal agent of cocoa black pod disease. The radial growth of four strains of *T. asperellum* was monitored for 30 days on PDA modified medium at six levels of $a_w$ (0.995-0.880), three values of pH (4.5, 6.5 and 8.5) and three incubation temperatures (20, 25 and 30°C). Whatever the strain, mycelial growth rate was optimal at water activities between 0.980 and 0.995, independently of incubation temperature and pH. All strains appeared to be very sensitive to $a_w$ reduction. In addition, all four strains were able to grow at all temperatures and pH values (4.5-6.5), highest growth being observed at 30°C and pH= 4.5-6.5.

Keyword: radial growth rate, water activity

Introduction

The cocoa tree (*Theobroma cocoa* L.) is cultivated for its economically important bean. It is among the most developed cash crops in Western and Central Africa, and its production represents 70% of the 3 million tons produced worldwide (ICCO 2003). The global rise in cocoa production is limited by many constraints, such as cocoa black pod disease. Different species of *Phytophthora* are known to cause this disease, and these species vary according to both their aggressiveness and the level of crop loss caused (Appiah et al., 2004). *P. megakarya*, which prevails in Cameroon, is the most aggressive of the four main species of *Phytophthora* causing black pod disease of cocoa, with crop losses ranging from 60 to 100% (Ndoumbé-Nkeng et al. 2004). Classically, cocoa growers use farming strategies, genetic strategies, and most often chemical strategies to minimise the impact of black pod disease (Akrofi et al., 2003).

Biological control by means of antagonistic microorganisms is an emerging strategy in many countries affected by this disease (Krauss & Soberanis, 2001). In Cameroon, research focusing on biological control of *Phytophthora megakarya* began in 1999. It has led to the isolation and identification of mycoparasitic strains of *Trichoderma asperellum* (Tondje et al., 2005).

Currently these *T. asperellum* strains (PR10, PR11, PR12, and 659-7) are applied under natural conditions in cocoa fields but the level of protection afforded by such biocontrol agent preparations has been inconsistent. The variable performance of *T. asperellum* as a biocontrol agent could be due to the influence of environmental factors that vary in time and from farm
to farm. No literature is available concerning the effect of these environmental parameters on the development of *T. asperellum*. In this context, the main objective of this work was to determine *in vitro* effects of temperature, pH and $a_w$ on the radial growth rate of *T. asperellum* strains (PR10, PR11, PR12, 659-7).

**Materials and methods**

**Microorganisms**

Four strains of *T. asperellum* (PR10, PR11, PR12 and 659-7) were used in this study. These strains were isolated from tubers of *Xanthosoma* spp. and *Musa* spp. in a mixed crop field around Yaoundé in Cameroon and then stored on PDA medium at 4°C.

**Medium**

The basic medium used was Potato Dextrose Agar (PDA, $a_w$=0.995). The $a_w$ was modified by adding increasing amounts of glycerol to obtain $a_w$ levels of 0.980, 0.960, 0.930, 0.910, and 0.880 at three different temperatures (Lahlali et al., 2005). The medium was buffered with 0.1 mM NaH$_2$PO$_4$, the final pH of the medium being adjusted to pH 4.5 with 80% H$_3$PO$_4$ or to pH 6.5 or 8.5 with 1 M NaOH before autoclaving. The $a_w$ of all media was measured with an AquaLab series 3 instrument (Decagon, 950 NE Nelson Court Pullman, Washington 99163).

**Preparation of the inoculum**

A 10-day-old colony culture of *T. asperellum* grown on PDA was used to obtain spore suspensions. Ten to twenty ml of sterile distilled water containing 0.05% Tween 20 was added to the Petri dish and conidia were carefully scraped from the surface of the colonies before filtration through sterile cheesecloth. Suspensions were adjusted to $10^6$ conidia/ml using a Bürker cell, then 10-µl aliquots of suspension were inoculated at the centre of Petri dish containing test medium. After inoculation, the Petri plates were sealed in polyethylene, and then incubated for 30 days at 20, 25 or 30°C.

**Data recording**

The radius of each growing mycelial colony was measured daily in two perpendicular directions, without opening the Petri dishes, until the plates were completely colonised. Four replicates were used for each combination of experimental conditions. The radial growth rate (mm day$^{-1}$) for each $a_w$, temperature, pH combination was obtained from linear regression slopes of the temporal growth curves.

**Statistical analysis**

Growth rates were subjected to the general linear model (GLM) procedure of the Statistical Analysis System (SAS). Statistical significance was performed at the $P \leq 0.05$ level. Where analysis revealed significant differences, Duncan’s multiple range test for separation of means was performed.

**Results**

Statistical variance analysis of the data provided a highly significant effect ($P<0.0001$) of $a_w$, incubation temperature, pH and their two and three interactions on mycelial growth rates of *T. asperellum* strains PR10, PR11, PR12 and 659-7 (results not shown). Duncan's multiple range test was performed to distinguish the various homogeneous groups relating to each factor studied here and for each strain (Figs 1-3). Regardless the strain, the growth rate was significantly correlated to the water activity of the medium. The highest value was observed at $a_w$ of 0.980, independently of incubation temperature and pH level. At $a_w$ of 0.910, strains PR10, PR11 and PR12 displayed a residual growth under some conditions. No growth was observed at $a_w$ of 0.880 whatever the strain.
At 30°C, the growth rates were highest for all strains. At 20°C, the growth rate was lowest as compared to 25-30°C. However no growth was observed at $a_w$ of 0.910 $a_w$ for strains PR10 and 659-7 whatever the level of pH tested. Also, a very low residual growth was observed for strain PR11. All strains were able to grow at all pH levels (4.5, 6.5 and 8.5). The growth rate was almost always higher at pH 4.5 for PR10, PR11 and 659-7 and at pH 6.5 for PR12 than pH 8.5.

**Discussion**

The statistical analysis of radial growth rate of *T. asperellum* showed that the environmental factors ($a_w$, temperature and pH) are a key elements could limited the development of the *T. asperellum* strains. The $a_w$ factor has the greatest influence on the growth rate of *T. asperellum* strains. The optimal growth was observed at $a_w$ ranging from 0.980 to 0.995. *T. asperellum strains* were shown to be highly sensitive to the dropped of $a_w$ of the medium. These results are in agreement with those reported by Kredics et al. (2004) who observed limited growth of *Trichoderma spp* at 0.92 $a_w$. Subsequently, applying *Trichoderma* strains as
biological control agent must be supported by a substrate that maintains a constant high $a_w$ to allow the growth of the propagules (Wakelin et al., 1999).

Our results indicated that the growth of the studied strains of *Trichoderma* was possible at all tested levels of temperatures and pH ranging from 4.5 to 8.5. The highest growth of *T. asperellum* strains was observed at incubation temperature of 30°C as previously reported by Samuels et al. (1999), and at pH values between 4.5 and 6.5. Similar results were found on other species of *Trichoderma*. Kredics et al. (2004) reported that *T. harzianum, T. aureoviride* and *T. viride* were able to grow within a broad range of pH ranging from 2.0 to 6.0 with an optimal growth at pH = 4.0. The lowest growth rate of the studied *T. asperellum* strains was recorded between 20 and 25°C at lower values of $a_w$. This result seems explained the inconsistent performances of the mycoparasitic strains of *T. asperellum* when they are applied in cocoa plantations in Cameroon. In fact, in cocoa plantations, one observes throughout the growing period, fluctuations of relative humidity and the average minimum and maximum daily temperatures recorded under cocoa trees are about 20 and 26°C respectively (Ndoumbè-Nkeng et al., 2004). Under these conditions, field test results showed strain PR11 to be most effective at all location (Tondje et al., 2005). In our study likewise, PR11 displayed faster

Figure 2. Effect of temperature on radial growth rate of *T. asperellum* strains

[Graphs showing radial growth rates at different temperatures for PR10, PR11, PR12, and 659-7]
growth than other strains at 20°C and low water activity (0.930). This resistance to stress may favour better colonization and faster establishment of the antagonistic population, and thus better effectiveness.

Figure 3. Effect of pH on the radial growth rate of *T. asperellum*

The results from the assessment of the effects of environmental factors such as temperature, $a_w$, and pH on the growth of *T. asperellum* strains on solid substrate is a first investigation making it possible in the future to predict the behaviour and to handle the ecophysiological aptitudes in order to develop an effective formulation for a biofungicide based on *T. asperellum*.

Acknowledgements

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References


Functional characterization of grape defence genes to improve the biocontrol activity of *Pseudomonas fluorescens* against *Armillaria mellea*

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**Abstract:** *Armillaria mellea* is the main causal agent of grape root rot in some important viticulture areas. It causes vigour decline and plant death. So far, no resistant *Vitis* rootstocks have been identified and the pesticides are ineffective in controlling this disease. Since young grapes do not show *A. mellea* symptoms during the first 3-4 years, the activation of defence mechanisms has been hypothesised. In order to study defence response at molecular level in the widely used rootstock (Kober 5BB), the suppression subtractive hybridization approach has been used and specific genes induced 24 h after *A. mellea* inoculation have been identified. To elucidate the function of these genes in the plant defence response, their full-length sequences have been obtained and cloned in a vector suitable for heterologous expression in bacteria. The characterization of antifungal properties of the recombinant proteins will identify grape genes involved in defence reaction against *A. mellea*. These antifungal genes will be mobilized in *Pseudomonas fluorescens* to introduce a new antagonistic trait in this biocontrol agent.

**Key words:** root rot

**Introduction**

Grapevine root rot, caused by the fungus *Armillaria mellea*, is a serious and increasing disease in some important grape growing areas (Baumgartner & Rizzo, 2002; De Luca et al., 2003). *A. mellea* infects grape roots and produces extensive fans of hyphae that spread under the bark decaying the underlying xylem. Infected plants show a decline in vigour, stunted shoots and leaves, dwarfed bunches and autumnal early change in leaf colour (De Luca et al., 2003). Diseased plants normally die some years after pathogen infection (Baumgartner & Warnock, 2006). The fungus rapidly spreads either through root-to-root contacts or through specific structures called rhizomorphs (Robinson et al., 2000). The rhizomorphs are provided with nutrients translocated from the infected tree and they can also grow beneath the bark of dead trees, remaining quiescent for a long time in the soil.

Since commercial pesticides are not effective to control *Armillaria* spp. infections (Aguin et al., 2006) and resistant *Vitis* rootstocks have not been identified (De Luca et al., 2003), the development of efficient control methods would be important to prevent the diffusion of the pathogen. The use of antagonistic microorganisms has been recently reported as a promising biological approach to control *Armillaria* (Cox & Sherm, 2006; Baumgartner & Warnock, 2006). In addition, field observation indicate that young vines do not show symptoms, suggesting an active defence mechanism. The characterization of the natural plant
defence reaction against \textit{A. mellea} and the selection of efficient biocontrol agents are important starting points to develop integrated control strategies and select resistant rootstocks. The functional characterization of some grape defence genes to be eventually introduced in \textit{Pseudomonas fluorescens} to increase antagonist activity against \textit{A. mellea}, is here presented.

\section*{Material and methods}

\textbf{Plant and fungal materials}

Rooted cuttings of the rootstock Kober 5BB were grown in greenhouse on stone wool (Grodan). \textit{A. mellea} (strain 7A, SafeCrop collection) was isolated from an infected vine in Piana Rotaliana (Trentino, Italy). \textit{A. mellea} was grown on malt extract agar at 20°C in the dark. Each root system of two Kober 5BB plants was sandwiched between two Petri dishes containing four weeks-old \textit{A. mellea} colonies. The contact was kept for 24 h at 25-28°C.

\textbf{Subtracted cDNA library construction and Differential screening of subtracted library}

RNA from infected and non-infected roots was extracted with the hot borate method (Moser et al., 2004) and suppression subtractive hybridisation (SSH, Diatchenko et al., 1996) was performed with the PCR-Select cDNA subtraction kit (Clontech). cDNA fragments were cloned into pCR2.1-TOPO vector (Invitrogen) and mobilised into \textit{E. coli TOP10} cells. Clones obtained by SSH were amplified by colony-PCR and spotted on Hybond-N+ membrane (GE Healthcare). Nebulin was spotted as constitutive internal control. Labeled cDNA probes coming from infected and non-infected roots were generated by DIG Labeling Kit (Roche) and membranes were hybridised.

\textbf{Full length amplification by 5´-RACE RT-PCR}

The full-length coding sequences of the selected genes were obtained with the 5´ RACE System (Invitrogen) with slight modifications. First strand cDNA was synthesized from RNA of \textit{A. mellea} treated roots using oligo-dT primer. After cDNA purification, homopolymeric tail was added to the 3’-end using TdT in presence of dCTP. The genes were amplified using specific primer that anneals at 3’ end of the gene and the oligo-dG anchor primer. Following purification the 5’ RACE products were cloned into pCR2.1-TOPO and sequenced.

\textbf{Expression of recombinant proteins}

New specific primers were designed to add the restriction sites for the cloning in the expression vector pET22b (Novagen) and the constructs were mobilised in \textit{Escherichia coli} BL21(DE3) (Novagen). Proteins overexpression was induced by adding IPTG to the culture and harvesting the cells after 6 h at 37°C. Total proteins were analysed by SDS-PAGE.

\section*{Results and discussion}

\textbf{Isolation of grape induced genes}

A subtracted cDNA library enriched in transcripts induced in the grape roots 24 h after \textit{A. mellea} treatment was constructed using SSH approach and about 2000 clones were collected. For validation the clones were screened by hybridisation with four different cDNA probes: tester (root + fungus), driver (root alone), tester minus driver and driver minus tester. Following this check, 56 clones were considered truly up-regulated and were then subjected to single pass sequencing. Fifty-one clones, which revealed good sequence information, were clustered into contigs in order to group the sequences with significant overlapping regions and thus belonging to the same cDNA sequence. This procedure defined 8 clusters and 19 singletons. These sequences were used to identify the corresponding tentative consensus (TC) using BLAST-N against the TIGR Grape database. Functional annotation of the TC using
BLAST-X against the non-redundant NCBI database revealed the presence of several defence-related genes, such as Protease inhibitors, Thaumatin and Tumour related protein (Table 1). The induction of a glutathione S-transferase suggests the production of reactive oxygen species, as observed in tobacco cells after *Armillaria* treatment (Vitecek et al., 2005).

The induction of A-03_7, A-08_2, B-03_4, D-05 has been also confirmed in young roots of in vitro grown Kober 5BB plants 24 h after *A. mellea* treatment by real time RT-PCR experiments (data not shown). Furthermore, plant protein extracts of young grapevine roots challenged with *A. mellea* for 110 h showed a 40% of growth inhibition of the *A. mellea* mycelia in solid medium compared to the extracts of untreated plants (data not shown). These results demonstrate that young Kober 5BB roots react to *A. mellea* infection, upregulating specific defence genes, but the functions of these genes in the plant defence response and their correlation with the antifungal activities of the root extracts remain unknown.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>TC</th>
<th>P-value</th>
<th>Protein homologue (Accession Number)</th>
<th>E-value</th>
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<tr>
<td>A-03_7</td>
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<td>Phase-change related protein (antimicrobial peptide) <em>Quercus robur</em> (CAB72442.1)</td>
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<td>A-12_6</td>
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<td>A10_3</td>
<td>TC38419</td>
<td>6.3E-27</td>
<td>Calmodulin 8 <em>Daucus carota</em> (AAQ63462.1)</td>
<td>3E-79</td>
</tr>
<tr>
<td>E04</td>
<td>TC38633</td>
<td>1.5E-77</td>
<td>Transcription factor <em>Vicia faba</em> (CABA6481.1)</td>
<td>1E-132</td>
</tr>
<tr>
<td>C05</td>
<td>TC45326</td>
<td>4.0E-45</td>
<td>Proline-rich cell wall protein <em>Vitis vinifera</em> (CAB85624.1)</td>
<td>2E-28</td>
</tr>
</tbody>
</table>

TC: tentative consensus; E-value: statistical alignment significance of NCBI BLAST-X; P-value: statistical alignment significance of TIGR BLAST-N.

**Cloning the full length coding sequence of some grape defence genes**

Using the RACE-PCR method, the full-length cDNA of the most interesting defence genes identified by SSH, were cloned (Fig. 1). The coding sequences of Chitinase 3 and Thaumatin 1 were cloned as control antifungal genes. Bioinformatic analyses of the isolated genes were carried out to confirm the homology to the corresponding genes of other species.

**Expression of recombinant grape proteins**

By adding new restriction sites, the full-length cDNAs were subcloned into the pET22b vector to express the recombinant proteins in the periplasmic fraction of *E. coli*. The protein expression experiments demonstrated a high production of recombinant Protease inhibitor and the need of optimization for the expression of the other proteins (data not shown). As future development, extract of bacteria containing the recombinant proteins will be used to test the effect on *A. mellea* growth in vitro, in order to examine their antifungal properties. The defence genes showing efficient antifungal activity will be sub-cloned and mobilised in *P.*
fluorescens. The transgenic *P. fluorescens* expressing the grape recombinant protein will be characterised for its antagonistic properties against *A. mellea*, using dual culture. Although nowadays this approach is far from any practical application, the final aim of this study is to explore the potential increase in biocontrol activity of transformed microorganisms.

Figure 1. Agarose gel electrophoresis of Kober 5BB; Chitinase 3 (2) and Thaumatin 1 (3) and RACE RT-PCR products of Protease inhibitor (4), Tumor-related protein (5), Thaumatin (6) Phase-change related protein (7), Proline-rich cell wall protein (8) and molecular marker (1).

Acknowledgements

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References


Survival of *Trichoderma atroviride* 122F on strawberry phylloplane and in soil

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**Abstract:** *Trichoderma atroviride* P. Karst. 122F was isolated from decayed hazelnut wood in northern Italy. Its antagonistic activity against *Armillaria mellea*, the causal agent of root rot in agricultural crops and forests, was observed in *vitro* and *in planta* tests. The objective of the research was to characterize the biology and ecology of this antagonist. To better characterise *T. atroviride* 122F, the influence of pH, temperature and nitrogen and carbon sources on its *in vitro* growth was evaluated. *T. atroviride* 122F survival was assessed on strawberry leaves under greenhouse controlled conditions and in three different soil types (sterilised and non-sterilised) at room temperature. *T. atroviride* 122F conidial suspension (1×10⁶ conidia/ml) was sprayed on leaves of four week old strawberry plants. Leaf samples were taken 0-45 days after spraying. CFUs were counted on *Trichoderma* sp. selective medium to assess *T. atroviride* 122F survival. *T. atroviride* 122F survival in soil was assessed in polypropylene bottles with an application rate of 1×10⁶ conidia/g soil. Assessments were carried out after 0-90 day after application. *T. atroviride* 122F survival in soil was measure as described before. It was found that isolate 122F is mesophilic, with an optimum growing temperature of 25°C. The fungus presents a wide pH tolerance, but growth was reduced on alkaline media (pH≥9). Nitrogen and carbon sources as yeast extract, peptone, tryptone, galactose, mannose and sucrose gave the highest mycelium biomass production (dry weight). The fungus can survive on strawberry leaves, but decreased from 5.23×10⁴ to 4×10² CFU/mm at the end of the experiment. *T. atroviride* 122F survives and multiplies in sterilised soils, whereas no increase, followed with a decrease after 20 days, was seen in non-sterilized soils, probably as a consequence of competition with soil microbial population. This hypothesis is also supported by differences in survival and establishment of *T. atroviride* 122F in the three non-sterilised soils. *T. atroviride* 122F is not highly demanding for growing and adequately survived under the tests conditions, showing a good potential for soil and foliar application.

**Key words:** biocontrol, persistence

**Introduction**

In mycological studies the growth and survive may be all that is important. Since fungi are being used for industrial and biotechnological purpose fermentations should be carried out under the best possible conditions of temperature, pH, and nutrition (Kendrick, 1992). Measurement of specific growth rate or biomass yield coefficient on different media or under different environmental conditions provides a characterization of the fungal physiology. Such information may be particularly important to technologists attempting to predict rates of substrate utilization or to optimize biomass production or product formulation (Prosser, 1991). Moreover, it is imperative to determine the survival and establishment of a potential biocontrol strain to new sites to ensure the most effective formulation and application.
strategies (Dodd et al., 2004). *Trichoderma atroviride* P. Karst 122F is a promising fungal agent for biological control against soil plant pathogens; *Armillaria mellea* (Vahl) P. Kumm in particular. This study reports nutritional and environment factors requirements for growth and evaluate survive of *T. atroviride* 122F on soil and strawberry leaf surface.

**Materials and methods**

The effect of temperature on mycelia growth was assessed in 90 mm Petri dishes containing PDA and inoculated centrally with an agar plug of 7-day-old cultures. The plates were incubated in the complete darkness at -1, 5, 10, 15, 20, 25, 30, 37 and 40°C from 30 days. The effect of pH on mycelium growth was assessed as done for temperature. Media pH was adjusted after autoclaving with sterile solutions of 1 N HCl or 2 N NaOH to obtain the following levels: 3, 4, 5, 6, 7, 8, 9 and 10. Colony diameter was recorded daily. Ten replicates plates were inoculated for each treatment and factor studied.

The effect of nutrient sources on mycelium dry weight was assessed in Czapek Dox liquid modified medium (Oxoid) amended with glucose (10 g/l) or with glycine (1 g/l) (1:1; w/w), when testing carbon sources (CS) and nitrogen sources (NS), respectively. Nutrient sources were filter-sterilized and added to autoclaved media at a rate of 2 g/l (NS) and 20 g/l (CS). Flasks containing 100 ml of media were inoculated with an agar plug containing mycelium from 7-day-old cultures for each nutrient source. After 13 days of incubation, the mycelium was harvested through filter paper, dried and the dry weight was recorded.

Survival on leaf surface was measured as CFU/ml. 10 strawberry plants were sprayed with a conidial suspension (10⁶ conidia/ml) until run off. A leaf disc (2.5 cm Ø) was cut from each plant leaf at 0, 1, 3, 7, 15, 30 and 45 days after inoculation. It was transferred into flasks containing 5 ml SDW + Tween 80, shaken for 3 min and left to stand for an additional minute. A dilution series in SDW was set up and the suitable dilution was plated on semi-selective media (PDA amended with 100 ppm rose Bengal, 100 ppm streptomycin and 50 ppm chloramphenicol). The plates were incubated at 25°C and CFU counts were made after 7 days.

Survival of *T. atroviride* 122F on soil was assessed in three soil types from North Italy (Table 1). 100 g Sieved (< 2 mm) soil was filled in polypropylene bottles and samples were left untreated (non-sterile) or autoclaved. Samples of each soil were assessed to provide physical and chemical characteristics. An inoculum dose of 1×10⁶ CFU.g⁻¹ soil was routinely used. The bottles were incubated at room temperature. Five replicates were set up for each soil type and soil state. The CFU/g soil were determined as described above at intervals of 0, 1, 5, 10, 20, 30 and 45 days.

**Results and discussion**

Temperature is one of the cardinal factors affecting fungal growth. The majority of fungi is mesophiles and grows in temperatures 5-35°C, with optima between 20 and 25°C (Dix & Webster, 1995). *T. atroviride* 122F is able to grow at 10–30°C, with an optimum temperature for growth of 25°C. Thirty days at 35°C killed the fungus, however after 30 days at -1°C conidia were still able to germinate. Latent period was shortest at 25°C and first conidia were visible on the third day.

*Trichoderma* strains grow usually optimal between pH 4 and 6.5, and only few *Trichoderma* spp. seem to tolerate pH<3 (Kubicek-Pranz, 1998). *T. atroviride* 122F presents a wide range of pH tolerance with an optimum on acid media (pH 4-6). Mycelium growth was
significantly reduced on alkaline medium (pH $\geq 9$) and conidiation is reduced at pH 3, and 8 and above.

As expected for a common soil saprophytic fungus, *T. atroviride* 122F was able to utilize a wide range of compounds as sole carbon and nitrogen sources. Fungal growth in media was significantly superior when it was supplied with some nitrogen sources such yeast extract, nitrite, tryptone, peptone, glutamine and asparagine or some carbon sources such mannose, galactose, sucrose, malt extract, cellobiose and glucose (Figs 1 and 2).

In general, fungi utilize a large number of organic compounds as a carbon source. About half of the dry weight of fungus cells consists of carbon, which gives an indication of the important role of carbon compounds within the cell. Despite that D-glucose is favored by more fungi than any other sugar, mycelium production of *T. atroviride* 122F was more extensive in media containing D-mannose, D-galactose and sucrose. Similar occurrence was observed by Aube & Cagnon (1969) for a *T. viride* strain that utilized D-Xylose, Sucrose and cellobiose better than D-glucose. Previous studies on carbon and nitrogen nutrition of *Trichoderma* show that there is a good deal of variation among species and indicate physiological differentiation of some strains and species. According to Manczinger & Polner (1985) the utilization of some carbon sources such as sucrose, lactose, maltose and others, is species dependent and can be used for chemotaxonomic purpose. The capacity of *T. atroviride* 122F to degrade and utilize cellobiose, a hydrolysis product of cellulose, is an important indication of its cellobiolytic enzyme production.

Bender (1975) examining polysaccharide content of culture filtrates of basidiomycete fungi, found that galactose and mannose were the major constituent sugars, amounting to more than 50% of the total. High utilization of these sugars by *T. atroviride* 122F may favor antagonism toward the basidiomycete *A. mellea*.

*T. atroviride* 122F was able to utilize simple as well as complex nitrogen sources for growth, and utilized organic better than inorganic nitrogen. The fungus used small peptides of up to six amino acid (yeast extract, peptone and tryptone) better than all others nitrogen sources. Although the response of different species to any single amino acid varies widely, asparagine most often gives good fungal growth, and this amino acid supported better growth of *T. atroviride* 122F than the other amino acids tested. Nitrate and ammonium ion support poor to moderate growth.

Table 1. Physicals and chemicals characteristics of three soils proceeding of North Italy used on experiment of *Trichoderma atroviride* 122F survive on static microcosm conditions

<table>
<thead>
<tr>
<th>Soil</th>
<th>Position</th>
<th>Texture</th>
<th>pH</th>
<th>C</th>
<th>N</th>
<th>$P_2O_5$ (mg/Kg)</th>
<th>$K_2O$ (mg/Kg)</th>
<th>MgO (mg/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N 46°11.873’</td>
<td>Clay loam</td>
<td>7.86</td>
<td>8</td>
<td>0.4</td>
<td>8</td>
<td>105</td>
<td>537</td>
</tr>
<tr>
<td></td>
<td>OE 11°08.312’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>N 46°11.930’</td>
<td>Sandy loam</td>
<td>7.53</td>
<td>74</td>
<td>3.5</td>
<td>38</td>
<td>158</td>
<td>801</td>
</tr>
<tr>
<td></td>
<td>OE 11°08.645’</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>N 46°11.964’</td>
<td>Sandy loam</td>
<td>7.38</td>
<td>84</td>
<td>3.9</td>
<td>72</td>
<td>336</td>
<td>678</td>
</tr>
<tr>
<td></td>
<td>OE 11.08,417’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Growth of *Trichoderma atroviride* 122F measured as mycelia dry weight (13-day-old cultures) in liquid medium with a specified carbon source. Data are means of five replicates. Values followed by the same letter are not significantly different at least $P=0.05$ according to Fisher significant difference test. Error bar = ± standard deviation of the means.

Densities of total conidia of *T. atroviride* 122F on strawberry phylloplane decreased quickly during the first week. The fungus population showed a continuous decline until the 14$^{th}$ day after application (Fig. 3), maintaining afterwards a few viable CFU until the 45$^{th}$ day when the experiment was concluded. This result is in agreement with the data reported by Freeman et al. (2004) on *T. harzianum* T39 survival on strawberry leaves.

The survival of the fungus in soil is noticeably influenced by the autoclaving treatment (Figs 4 A B). In sterilized soil its population was increased, whereas in non sterile soil the CFU remained at the same level in soil 2 and 3. Probably the presence of indigenous
competitors in natural soil is the cause of a lack of increase observed in non sterile soil. In soil 1, the non sterilisation still allowed a small CFU increase.

Figure 3. Survival of *Trichoderma atroviride* conidia on strawberry leaf. Data are means of ten replicates and were transformed by log(×).

Figure 4. Survival of *Trichoderma atroviride* 122F on autoclaved soil (A) and non-sterile soil (B). Data are means of five replicates and were transformed by log (×).

**Acknowledgements**

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References


Microcosm approach for examining the survival and migration of *Trichoderma atroviride* 122F in soil

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**Abstract:** Survival and dispersal of a biocontrol agent in the soil is an important aspect both for increasing its control efficacy and in risk assessment. Much of this information is usually obtained with the use of microcosms. An undisturbed soil structure is an important prerequisite for representing natural environmental conditions. A new microcosm prototype is proposed in experiments using an “intact soil” core. Microcosms were manufactured from iron pipes (7 cm inside diameter) cut to 50 cm lengths. The pipe was perforated every 10 cm over length for easily sampling soil at increasing depths. A preliminary experiment was run in December 2005 to optimize sampling frequency and extracted soil sample size. A second experiment was set up to evaluate the movement with percolating water of *Trichoderma atroviride* P. Karst 122F. Soil cores were taken from the field in March and May 2006, by inserting the pipe for 50 cm into the soil and gently removing it without disturbing the sampled core. The microcosms were immediately placed in an incubator at 20°C. The surface was irrigated according to the natural rain quantities. Conidia of *T. atroviride* 122F grown on rice were inoculated on the microcosm’s soil surface by mixed the suspension with the first centimetre of soil (final conidia concentration of $1 \times 10^6$ CFU/g soil). A soil sample was weekly removed through each perforation representing a particular microcosm soil layer. Dilution plating was carried out using a semi-selective medium for *Trichoderma* spp. to count the recovered *T. atroviride*’s CFUs during time. A good survival was seen in the first layer of soil and slow and limited movement with percolating water was seen. These experiments, after validation, can provide information on possible behaviour and fate of soil application of *T. atroviride* 122F.

**Key words:** biocontrol agent, risk assessment

**Introduction**

The success of *Trichoderma* strains as BCAs is due to their high reproductive capacity, ability to survive under very unfavourable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defence mechanisms in plants. Good antagonists are usually able to overcome the fungistatic effect of soil that results from the presence of metabolites produced by other species, including plants, and to survive under very extreme competitive conditions (Benitez et al., 2004). *Trichoderma atroviride* P. Karst is a biocontrol agent of a wide range of economically important aerial and soil-borne plant pathogens. The mycoparasitic activity of this organism is attributed to a combination of successful nutrient competition, the production of cell wall-degrading enzymes and antibiosis. The availability of a versatile expression system for *T. atroviride* based on application of recently characterized biocontrol-related promoters suggested that it might be possible to improve the disease control effect of this biocontrol agent (Brunner et al., 2005). In the development of a biocontrol agent for commercial application, it is important to assess its potential survival and dispersal in new environment prior to release it at large scale. Such information is imperative.
to ensure the most consistent and reliable disease control and to assess risks for humans and environment. Little is known about the survival, spread and general population dynamics of *T. atroviride* strains once they have been applied to the field (Dodd et al., 2004). Ideally, pilot studies can be performed in microcosms that represent a complex system containing the ecological network from which an organism was obtained. There are several types of microcosms designs that vary from a few grams of soil in a bottle to a large chamber where many of the environmental conditions can be controlled. To test microorganism release into the soil, two types are mainly used: small amounts of sieved soil in a bottle and intact soil-cores. Intact and sieved-packed soil microcosms have been compared for vertical dispersal of microorganism (Gagliard et al., 2001). There is general consensus that intact soil-cores are useful for the pre-release testing of microbes to the soil, mainly because they contain the complexity and native biota found in the field.

With the aim of developing a standard intact soil core microcosm that is cheap and simple and can simulate field conditions, we evaluated the survival and vertical migration of a potential biocontrol agent *T. atroviride* 122F on a loam sandy soil. Survival and vertical dispersal of strain F122 was compared with constant water application and simulation of the natural rain during May-July in S. Michele all’Adige (Italy).

**Material and methods**

**Preparation of static microcosms**

Soil samples were taken from a vineyard in S. Michele all’Adige in 2005. Sieved soil was placed in 10 polypropylene boxes (100 g/box). Five replicates (boxes) were autoclaved prior inoculation. The boxes were inoculated with *T. atroviride* F122 using $1 \times 10^6$ Colony Forming Units (CFU)/g soil. The boxes were incubated at room temperature. Assessments were carried out immediately and at intervals of 1, 5, 10, 20, 30, 60 and 90 days after inoculation. A dilution series was done in sterile deionised water (SDW) and plated onto PDA amended with rose Bengal (100 ppm), streptomycin (100 ppm) and chloramphenicol (50 ppm). The plates were incubated at 25°C and final colony counts were made after 7 days.

**Preparation of soil-core microcosms**

Microcosms were manufactured using iron pipes (7 cm inside diameter) cut to 50 cm lengths (Fig. 1). The pipe wall was perforated every 10 cm of length to allow easily sampling of soil at five levels (0, 10, 20, 30, 40 cm). Soil cores were taken from the same vineyard mentioned above in March and May 2006. Vegetation was first removed from the surface of the sampling area. Pipes were driven 50 cm into the ground and gently removed by hand without disturbing the soil core (intact soil core microcosms). The microcosms were immediately placed upright into an incubator at 20°C. Three replicates were inoculated with *T. atroviride* 122F and three replicates were kept untreated.

**Microorganism and inoculum**

*T. atroviride* 122F was isolated from decayed wood and maintained in PDA media at 4°C. The fungus culture was grown in sterilized boiled rice for 21 days at 25°C. An inoculum dose of $1 \times 10^6$ cfu/g soil was routinely used. The conidia used as inoculum showed an average viability close to 100%. Microcosms were inoculated within 24 h after their establishment by mixing the top 1 cm of soil layer with the inoculated rice.

**Sampling procedure**

At each sampling time a small sample of soil (0.5 g) was taken from each level of the microcosm soil profile and placed in sterile flask containing 5 ml of SDW + 0.01% Tween 80, mix by vortexing for 1 min and left to stand for 3 min. A dilution series in SDW was done and plated onto semi-selective media. CFUs were counted after 7 days of incubation at 25°C.
Soil samples were taken just after the application of *T. atroviride* and once a week for 5 weeks for the experiment started in March, and at time 0, 1, 2, 5, 9 weeks for the experiment started in May.

Figure 1. Soil-core microcosms: Material used (left) and microcosms in incubation chamber (right), visible are the holes for taking the samples.

**Rain simulation**

Water was added to microcosm in two different ways (Table 1). For the first experiment realized in March, 104 ml of water were added weekly on the surface of each microcosm, quantity corresponding to the average of rain during May in the last ten years in S. Michele all’Adige (total of 96 mm). For the second experiment in May 2006, the water was added according the quantity and timing of the natural rain in the same location (75 mm during the first 4 weeks). The experiment was run until the 9th week (total of 330 mm of water added).

**Results**

*T. atroviride* 122F CFUs counts in static soil microcosm showed that the fungus is able to survive and in the tested soil for at least 90 day with, maintaining the same conidia concentration of initial inoculum (Fig. 2).

Table 1. Different quantities of water (mm) weekly added on each set of soil-core microcosms to simulate a constant and natural rainfall

<table>
<thead>
<tr>
<th>Week</th>
<th>Microcosm (I) added water (constant according average rain in May)</th>
<th>Microcosm (II) added water according natural rain in 2006</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>11.8</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>32.6</td>
</tr>
<tr>
<td>7</td>
<td>21.6</td>
<td>3.4</td>
</tr>
</tbody>
</table>
Figure 2. Survival of *Trichoderma atroviride* 122F on autoclaved soil (st) and non-sterile soil (ns). Data are means of five replicates and were transformed by log (x). Error bar = ± standard deviation of the means.

Figure 3. Survival and vertical migration of *Trichoderma atroviride* 122F into intact soil core microcosm on March/2006 (top) and on May/2006 (bottom). *W*=week. Data are averages of three replicates transformed by log (x+1). Error bar = ± standard deviation of the means.
In the microcosms not inoculated with *T. atroviride* 122F 1 to 3x10² CFU/g dry soil were counted, however none of the CFU was identified as *T. atroviride* (date not shown), which confirms that FI22 was not already present on and in the soil and that no cross contamination occurred.

On and in the inoculated microcosms the fungus grows rapidly colonizing the soil surface (layer 0). At the end of May experiment (9 weeks) the CFU concentration is higher than originally inoculated CFUs and similar to the initial inoculated CFUs at the end of the experiment of March (4 weeks). Variability between results of the two experiments can be attributed to the different relative humidity of the soils. Vertical migration was observed since the first week after *T. atroviride* F122 application on soil surface. At this depth the fungus was able to colonize the soil with a CFUs concentration of about 50% of CFUs on the surface. In the others layers the fungus concentration was lower, indicating that the fungus vertical migration and its colonization is mainly restricted to the area above 20 cm of soil depth (Fig. 3).

*T. atroviride* 122F presents a good survival on both experiments and this behaviour confirms what premise on literature by several *Trichoderma* species. The microcosm design was easy and cheap for analysing survival and dispersion in soil of *T. atroviride* F122. The microcosm results have to be validated by correlating them to the real fungus’s dynamic under field conditions. No major differences were seen between the patterns of dispersal of the microorganism in the two experiments performed, demonstrating that with same constant temperature there are no major changes in soil colonization by *T. atroviride* F122 between constant and “rain simulating” addition of water.

**Acknowledgements**

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**References**


Effects of allyl-isothiocyanate released by *Brassica carinata* meals on *Trichoderma* spp. and soil-borne pathogens

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Abstract: Biofumigation by *Brassicaceae* green manure or seed meals is a promising, ecological alternative to methyl-bromide against soil-borne pathogens, based on the release of glucosinolate-derived compounds toxic for several pathogens. The effects on the beneficial soil microflora, naturally occurring or artificially introduced as biological control agents, need to be elucidated. Forty strains of *Trichoderma* spp., a well known fungus used as biological control agent, were *in vitro* tested for tolerance to allyl-isothiocyanate, the biocidal volatile compound released by wetted *B. carinata* meal. The results showed variable responses among the *Trichoderma* isolates, highlighting fungicidal or fungistatic effects, while a fungicidal effect was found towards the pathogens (*Rhizoctonia solani* and *Pythium ultimum*) at the same dose. One isolate, Ba15 strain (*T. koningii*), showed a peculiar behaviour, tolerating biofumigation very well. Moreover it was able to help the growth of other *Trichoderma* isolates growing in presence of the meal in the same Petri dishes, overcoming the fungistatic effect, maybe partially detoxifying the biocidal molecule. This effect was not observed towards the pathogens, which still were killed even in presence of Ba15 strain. *Trichoderma* strains were also assayed for *in vitro* antagonism towards the pathogens, by the dual growth method, with differential responses. Ba15 strain did not evidenced any antagonism ability. In conclusion these findings seem to confirm that *Trichoderma* is generally less sensitive than the tested pathogens to biocidal compounds released by wetted *B. carinata* seed meal, suggesting a possible combined use, and that the addition of Ba15 strain into soil could favour the survival and growth of natural or introduced *Trichoderma* antagonists, protecting them from depressing effects.

Key words: myrosinase, phytopathogenic fungi, plant disease

Introduction

Biofumigation by means of *Brassicaceae* green manure or seed meals incorporation into soil is a promising, environmentally friendly alternative to chemical fumigation by methyl-bromide against soil-borne pathogens. This biological approach is based on the release of glucosinolate-derived toxic compounds, mediated by endogenous myrosinase from *Brassicaceae* disrupted tissues or seed meals, in presence of water (Brown & Morra, 1997). Several cases are reported in literature referring about the different efficacy shown by this approach towards many plant pathogens (Kirkegaard & Matthiessen, 2004). One point which still needs to be elucidated concerns the effects of the biocidal compounds, mainly isothiocyanates, on the beneficial soil microflora naturally occurring or artificially introduced as biological control agents (BCA).

This study aimed at investigating the effects of allyl-isothiocyanate released by *Brassica carinata* defatted seed meal, which contains sinigrin as major glucosinolate, towards *Trichoderma*, a beneficial fungus, utilised as BCA against several diseases, due to its features of antagonism, mycoparasitism, antibiosis and resistance induction (Harman et al., 2004).
Materials and methods

Defatted *B. carinata* seed meal (Biofence, Triumph Italia S.p.a) containing mainly sinigrin at 151 µmol g⁻¹, was utilised to test *in vitro* *Trichoderma* tolerance to biocidal volatile compounds (mainly allyl-isothiocyanate) released upon meal wetting.

Forty *Trichoderma* strains differing for species and origin (Table 1) were grown on Potato Dextrose Agar (PDA), then one plug from an actively growing colony was transferred in 90 mm four-sector Petri dishes on PDA, three different strains for each dish, three replicates. *B. carinata* meal was confined in the forth sector to avoid direct contact with *Trichoderma*. The dose of 66 mg of meal corresponding to 10 µmol of sinigrin was chosen on the base of previous experiments. The dishes were sealed with parafilm immediately after wetting the meal with 150 µl of distilled water, to avoid loosing volatile compounds. Controls without meal were included. The same protocol was used for testing the meal effect on pathogenic strains of *Pythium ultimum* and *Rhizoctonia solani*.

*Trichoderma* strains were also assayed for *in vitro* antagonism towards the pathogens, by the dual growth method, growing *Trichoderma* and pathogens on PDA in the same 9 cm Petri dish and evaluating the ability of *Trichoderma* to grow and sporulate over the pathogens.

Results and discussion

Among the forty *Trichoderma* strains assayed, only Ba15 isolate (*T. koningii*) showed high tolerance to biocidal volatiles released by wetted *B. carinata* meal in a sealed environment, being able to grow already after 48 h. The majority of the strains were able to grow after dish opening on the 7th day, with variable lag phase, from 1 to 3 days (fungistatic effect) (Table 1). A fungicidal effect was instead observed for 3 strains, 3B24, Ba13 and Lt31 which were still unable to grow even after removal of parafilm, as observed also for both pathogens (data not shown).

Moreover, Ba15 strain allowed the growth of other *Trichoderma* strains in presence of allyl-isothiocyanate when put together in the same Petri dishes, showing the ability of somehow detoxifying the biocidal molecules released by the meal. This effect was equally observed for strains which had undergone fungistatic (Ba18, B41, B57, J56, Ba19, N2 J23, B13, P5, B51, Ba21) or fungicidal (Lt31) effects when were grown in absence of Ba15 (Table 1). The same effect was not observed towards the pathogens, which still were killed by meal toxic volatiles even in presence of Ba15 strain.

The dual growth method for antagonism assay showed differential ability of colonising *R. solani*: most strains could colonize and sporulate over the pathogen, except for Ba15, Ba19, B57, Regione, Ba24 and Lt36 (Table 1). Some isolates also exhibited a dark band in the contact zone between the colonies. All the strains were able to colonise and sporulate over *P. ultimum*, except for Ba19 and Ba15 strain. The last one also remained confined by the pathogen. In conclusion these findings seem to confirm that *Trichoderma* is generally less sensitive than the tested pathogens to biocidal compounds released by *B. carinata* seed meal, at the tested dose in a confined environment.

Since *B. carinata* meal showed a fungicidal effect towards some *Trichoderma* strains assayed, it cannot be excluded that its incorporation into the soil could affect endogenous *Trichoderma* population dynamics. Once toxic compounds have volatilised, endogenous or exogenous strains which have undergone only a fungistatic effect could begin to grow, exerting their beneficial effect. This finding opens good perspectives for a combined use of the biological control agent *Trichoderma* and the biofumigation technique through *B. carinata* meal incorporation into soil.
Table 1. Matrix and year of isolation, antagonism (dual growth method) towards *R. solani* and *P. ultimum*, and effect of the volatiles released by *B. carinata* meal on the growth of the 40 *Trichoderma* strains assayed

<table>
<thead>
<tr>
<th><em>Trichoderma</em> strain</th>
<th>Isolation matrix</th>
<th>Year</th>
<th>Antagonism class towards R. solani</th>
<th>R. solani</th>
<th>P. ultimum</th>
<th>Effect of volatiles released by B. carinata vs Trichoderma*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba15</td>
<td>Sugar beet soil</td>
<td>1986</td>
<td>C</td>
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<td>Ba15 presence: None</td>
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<td>A1</td>
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</tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>A1</td>
<td>Fungistatic</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>P8</td>
<td>Potato rhizosphere</td>
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<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
</tr>
<tr>
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<td>A1</td>
<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
</tr>
<tr>
<td>Lt1/05</td>
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<td>2005</td>
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<td>A1</td>
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<td>nd</td>
</tr>
<tr>
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<td>Sugar beet soil</td>
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<td>A1</td>
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<td>nd</td>
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<td>A1</td>
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<tr>
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<td>1986</td>
<td>A1</td>
<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
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<tr>
<td>K15</td>
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<td>A1</td>
<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
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<tr>
<td>Lt36</td>
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<td>C</td>
<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
</tr>
<tr>
<td>Ba8</td>
<td>Sugar beet soil</td>
<td>1986</td>
<td>A1</td>
<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
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<tr>
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<td>A2</td>
<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
</tr>
<tr>
<td>Regione</td>
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<td>-</td>
<td>C</td>
<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
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<tr>
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<td>A1</td>
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<td>nd</td>
</tr>
<tr>
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<td>Brassica juncea  rhizosphere</td>
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<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
</tr>
<tr>
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<td>Brassica nigra  rhizosphere</td>
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<td>B2</td>
<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
</tr>
<tr>
<td>J27</td>
<td>Brassica juncea  rhizosphere</td>
<td>2001</td>
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<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
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<tr>
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<td>Sugar beet rhizosphere</td>
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<td>Fungistatic</td>
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<tr>
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<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
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<td>J21</td>
<td>Brassica juncea  rhizosphere</td>
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<td>A1</td>
<td>Fungistatic</td>
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<td>nd</td>
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<tr>
<td>S2</td>
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<td>nd</td>
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<td>A1</td>
<td>Fungistatic</td>
<td>nd</td>
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<tr>
<td>Bf2</td>
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<td>A1</td>
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<td>nd</td>
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<tr>
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<td>A1</td>
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<td>nd</td>
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<tr>
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<td>A2</td>
<td>A1</td>
<td>Fungicidal</td>
<td>None</td>
</tr>
</tbody>
</table>

Antagonism class: A, B, C: complete, uncomplete or none colonisation of the pathogen, respectively; 1, 2: light or heavy browning of the pathogen, respectively.

*Evaluated after removal of parafilm.

nd: not determined
Finally the peculiar behaviour of Ba15 strain, which did not show any antagonistic ability towards the pathogens but was able to permit the growth of other *Trichoderma* strains in presence of the meal, would suggest the possibility to add it into the soil in order to favour the survival and growth of natural or introduced *Trichoderma* antagonists, protecting them from depressing effect exerted by *B. carinata* meal. Its activity seems to be linked to the presence of the meal since no stimulation activity was observed towards other *Trichoderma* strains when grown in the same Petri dish in absence of the meal.

Further investigation are needed to elucidate the mechanisms of detoxification exerted by Ba15 strain, which could be linked to the emission of volatile compounds able to biochemically interact with the isothiocyanates released by the meal or to the ability to trap them inside the mycelium, lowering the concentration of the biocidal compounds to levels not toxic for *Trichoderma*, but still lethal for the pathogens.

References


Microbial activity for a sound environment – field results from bacterial inoculation in potatoes and vegetables

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Abstract: The aim of the research carried out within the MASE-programme - Microbial Activities for a Sound Environment - is to supply background knowledge that will facilitate development and use of microbial based products in food and feed production. MASE, like the sister programme DOM – Domestication of Microorganisms – aiming at accumulating knowledge and support to the biotechnology industry regarding the fermentation, formulation and safety assessment of "non-conventional" microorganisms, both have the Swedish Foundation for Strategic Environmental Research – MISTRA – as a main funder. The MASE research activities are carried out in close collaboration with industrial partners and are focusing on six main areas: environmentally sound production of vegetables, potatoes, golf courses, cereals, sugar beets, and on biopreservation of food and feed with the use of microorganisms.

Field experimental research within the MASE vegetable and potato projects, were performed mainly in the south of Sweden and in Spain. Between two and eight bacterial isolates were fermented and inoculated to seeds, tubers or roots in 42 full scale field experiments during 2004 and 2005. Seed inoculations were performed in spinach, carrots, dill and peas. Root inoculations were performed in iceberg lettuce, broccoli, cabbage, kale, swedes, peppers and tomato. The treated plants in most of these tested crops showed more enhanced emergence and a more rapid growth than non inoculated controls. Since a rapid emergence often meant an advantage over diseases and weeds, this also often led to significant yield increases. The obtained yield increases could be explained either by direct plant growth promotion and/or by biological control of diseases. Based on the results obtained we see a good potential for utilising the tested bacteria in practical plant production. The fact that some of the bacterial isolates regularly induced a positive effect in several of the tested crops, also point to good economic possibilities and commercial potential for developing these as product ingredients. However, unpredictable factors here are costs for registration and for large scale commercial production, factors that are presently researched within the sister DOM-programme, and in collaboration with MASE industrial partners.
Biocontrol mechanisms in *Pseudomonas fluorescens* CHA0 depend on the nutrient status of the pathogen

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**Abstract:** Different biocontrol mechanisms are known to be essential for the biocontrol activity of pseudomonads. In strain CHA0 the production of the antimicrobial compounds e.g. 2,4-diacetylphloroglucinol (DAPG) is one of the most important biocontrol mechanisms. *P. fluorescens* CHA0 is able to protect cress against *Pythium ultimum* in a gnotobiotic system, where the pathogen is applied as mycelium covered millet. In this microcosm, a *gacA* mutant deficient in the production of various secondary metabolites such as DAPG, pyoluteorin and HCN is not able to protect the plants. Therefore, the production of the antimicrobial compounds is crucial for the biocontrol activity. We developed a new gnotobiotic system in which *P. ultimum* was added after cultivation on the clay mineral vermiculite with little nutrients. In this microcosm, a *gacA* mutant was able to protect cress against *P. ultimum* to a similar extend as the wild-type strain CHA0. Therefore, other mechanisms than the production of secondary metabolites seems to be important to control *P. ultimum* in this microcosm. This suggests that the biocontrol mechanism responsible for disease suppression is dependent of the nutrient status of the pathogen.
Influence of application time on survival, establishment and ability of *Clonostachys rosea* to control *Botrytis cinerea* conidiation on rose debris

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**Abstract:** The influence of application time (9, 12, 15 and 18 h) and the length of exposure to natural sunlight (0, 0.5, 1, 2, 4 and 8 h) on survival, establishment, and ability of *Clonostachys rosea* to suppress *Botrytis cinerea* conidiation on senescing rose leaves were investigated. The experiments were carried out in a climate-controlled greenhouse (Exp. 1) and in a plastic-covered greenhouse (Exp. 2). The conidia germination was significantly increased in the treatment kept on shadow and negatively correlated with application time from 9 to 18 h. The recovery of viable conidia from leaves reduced exponentially with length of exposure to sunlight. However, germination incidence was inversely proportional to the application time, independently of exposure to sunlight. These findings indicate that other factors beside solar radiation influenced germination. The relative humidity (RH) in the hours following inoculation correlated positively with germination, independently of sunlight exposure. Colonization of tissues by *C. rosea* was significantly reduced (40 to 50%) as exposure to sunlight increased. Despite the drastic effects of exposure to sunlight on *C. rosea*, the suppression of *B. cinerea* conidiation was only marginally affected (suppression of 94.5 to 100% and 65 to 93% at exp. 1 and exp. 2, respectively). Exposure of conidia to sunlight on the phylloplane for several hours reduced the efficiency of *C. rosea* in colonizing tissues, but only partially affects its ability to suppress *B. cinerea*. These results show the ability of *C. rosea* to withstand adverse environmental conditions and still provide suppression of *B. cinerea* conidiation on rose debris. Although biocontrol was still effective, we recommend that the application of the antagonist to crops should be done preferentially during periods of low sunlight and high RH, in order to maximize the pathogen suppression.

**Key words:** biological control, *Gliocladium roseum*, grey mould, *Rosa* sp., UV radiation

**Introduction**

*Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert and W. Gams (ex. *Gliocladium roseum* Bainier) was selected as an efficient antagonist against the grey mould pathogen *Botrytis cinerea* on roses (Morandi et al., 2003). The effectiveness of *C. rosea* is attributed to its ability in colonizing senescing and dead tissues faster than the pathogen and suppressing its conidiation. This ability, although inherent, is affected by several factors, including developing stage of host organs, inoculum concentration, time of application, and microclimatic conditions (Sutton et al., 1997). Earlier studies indicated that *C. rosea* is highly flexible regarding microclimatic conditions required by the fungus for colonizing rose debris and reducing conidiation of *B. cinerea* (Morandi et al., 2003, 2006).

Another factor influencing the performance of the biocontrol agent is the susceptibility to ultraviolet (UV) radiation on leaf surfaces. It is known that the long wavelength UV, especially the spectral portions of UV-B (280-310 nm) and UV-A (320-400 nm) present in natural sunlight can reduce both longevity of fungal spores and phylloplane colonization
As a result, the reduction in viability of the biocontrol agent increases the cost of the process due to the need for frequent applications and the use of protective additives in formulations (Ragaei, 1999).

Although *C. rosea* has been reported to be an effective antagonist against *B. cinerea* and other pathogens in many crops, the ecology of the fungus on the aerial plant parts, especially its tolerance to sunlight, has received little attention (Sutton et al., 1997; Hoopen et al., 2003). Therefore we evaluated the influence of application time and the exposure to natural sunlight on survival, establishment, and ability of *C. rosea* to suppress *B. cinerea* conidiation on senescing rose leaves.

**Material and methods**

Two experiments were conducted in two different locations. The first was conducted in a climate-controlled greenhouse at the Environmental Biology Department of University of Guelph, Ontario, Canada, whereas the second was in a plastic-covered greenhouse without climatic control at Embrapa Environment, Jaguariúna, São Paulo, Brazil. The air temperature, relative humidity (RH), and global solar irradiance were monitored during the experiments. Green fully expanded leaves of ‘Sonia’ and ‘Nivea’ plants were used in the first and second experiment, respectively. The leaves were detached to simulate senescing, washed on tap water, and superficially disinfested.

In the first experiment, each leaflet was inoculated by placing a 10 µl droplet of *C. rosea* inoculum (10^7 conidia ml^-1) on five sites that were approximately equidistant from each other and from the leaflet margin at each application time (9, 12, 15 and 18 h). Half of the leaves were kept in a black plastic covered area and the other half was exposed to sunlight. In the second experiment, a suspension of *C. rosea* (10^7 conidia ml^-1) was applied to entire leaves by means of an air-pressurized hand sprayer. The inoculated leaves were kept exposed to sunlight inside the greenhouse for 0 (check), 0.5, 1, 2, 4 and 8 h from 8 am to 4 pm. The inoculated leaves were kept inside the greenhouses until the next morning.

To estimate germination incidence of *C. rosea* (Exp. 1), 1-cm-diameter leaf disks (25 disks per treatment) were mounted in lactophenol plus 0.05% trypan blue on microscope slides and examined on a compound microscope. Germination was estimated on 100 conidia on each disk. A conidium was considered germinated when length of the germ tube exceeded the greatest diameter of the conidium. In the second experiment, the leaflets were shaken for 10 min in 50 ml water plus Tween 80 (0.05% v/v). From each suspension, one aliquot of 10 µl was placed on the center of a PDA plate and distributed over the surfacing using a bent glass rod. After four days at 25°C, the number of colony forming units (cfu) of *C. rosea* was counted.

To estimate *C. rosea* conidiation, 1-cm diameter leaf disks were removed from the inoculated sites at each leaflet (Exp. 1) or from the entire leaflets (Exp. 2) and transferred to paraquat-chloramphenicol agar (PCA) medium. There were five replicate plates with 10 disks per treatment. Conidiation was estimated after incubation at 25°C for 10 days through an eight-category scale (Morandi et al., 2001). To estimate suppression of *B. cinerea* conidiation, the disks were challenged with the pathogen (one 10 µl droplet of 10^5 conidia ml^-1) before placed in PCA. The control consisted in leaf disks inoculation with *B. cinerea* but not with *C. rosea*. Conidiation of *B. cinerea* was estimated after incubation at 25°C for 10 days through an eight-category scale (Peng & Sutton, 1991). Conidiation suppression (%) was calculated in relation to the check. Each experiment was set in completely randomized design and repeated once. Analysis of data of the two experimental repetitions invariably resulted in treatment effects in the same significance classes. Accordingly, data of one repetition are presented.
Results and discussion

*C. rosea* conidia germinated significantly more on treatments kept under shadow than under sunlight on all application times (Exp. 1). However, the germination incidence was inversely proportional to the application time, independently of exposure to sunlight, indicating that other factors beside solar radiation influenced conidia germination ability. The number of CFU recovered from inoculated leaves (Exp. 2) was exponentially reduced with the increase of exposure time (CFU=42.46*exp[-0.2224*h]; $R^2=0.88$). On treatments exposed to sunlight, the radiation correlated negatively with germination ($r=-0.94$). The RH was positively correlated with germination independently of exposure to sunlight ($r=0.95$ and $r=0.97$, for shadow and sun, respectively). This result is in accordance with previous finding that RH is a main factor on the establishment of *C. rosea* on rose debris (Morandi et al., 2006).

The exposure of the conidia of *C. rosea* on the phylloplane for several hours to a direct sunlight reduced their viability and efficiency in colonizes tissues. On both experiments, the area of the leaf disks colonized by *C. rosea* was significantly reduced with the increase of exposure time to sunlight after inoculation (Fig. 1A and C). However, in the first experiment the percentage colonized area values were higher than in the second experiment.

![Figure 1.](image)

Figure 1. *C. rosea* conidiation and suppression of *B. cinerea* conidiation on rose leaves inoculated with the antagonist at different times of the day (9, 12, 15 and 18 h) inside a climate-controlled greenhouse (A and B) or exposed to sunlight for 0 (control), 0.5, 1, 2, 4 and 8 hours inside a plastic-covered greenhouse (C and D). Evaluations were carried out after 10 days of incubation on PCA. Means followed by the same small letters or capital letters are not significantly different using Tukey test at 5%.

Despite reductions in *C. rosea* conidiation, there was no effect of application time on suppression of *B. cinerea* in experiment 1 (Fig. 1B). Although there was some reduction in
suppression with initial exposure to sunlight in experiment 2 (from 93 to 65% from no sunlight to 1 h sunlight), there was no reduction in suppression with additional sunlight, since no changes from 1 to 8 h, and only small, no significant change from 0.5 to 1 h sunlight was observed (Fig. 1D). Suppression of *B. cinerea* was still quite effective through all levels of exposure to sunlight (65-84% suppression from 0.5-8 h sunlight). These results show the ability of *C. rosea* to withstand adverse environmental conditions and still provide suppression of *B. cinerea* conidiation on rose debris.

Although biocontrol was still effective, we recommend that the application of the antagonist to crops should be performed preferentially during periods of low sunlight and high humidity, in order to maximize the pathogen suppression. In conclusion, these findings support the importance in studying ecological attributes of biocontrol agents to avoid failure and maximize their efficiency in the field (Köhl & Fokkema, 1998; Sutton et al., 1997).

**Acknowledgements**

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**References**


The effect of root exudates of tomato plants inoculated with biocontrol and/or arbuscular mycorrhizal fungi on the development of soil-borne tomato pathogens

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Abstract: The development of the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) in the presence of tomato root exudates was studied. Root exudates were extracted from tomato plants co-inoculated and inoculated separately with a commercial arbuscular mycorrhizal fungal inoculum and *Trichoderma harzianum*, *T. viride* or *T. atroviride*. In order to elucidate changes of the root exudation in plants treated as mentioned above, fungal growth of *Fol* was determined in a bioassay.

Keywords: biological control, plant diseases, plant-fungus interaction

Introduction

Arbuscular mycorrhizal fungi (AMF) as well as *Trichoderma* spp., although using different ways of action, are known to generate plant protection against soil-borne pathogenic fungi. The aim of this study was to elucidate if there are differences in the fungal growth of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) in root exudates of tomato plants inoculated with *Trichoderma* spp. and/or AMF. Furthermore, the influence of *Trichoderma* spp. on the root colonization of AMF was studied. These results could give an idea if the interaction of AMF and *Trichoderma* spp. is adverse, synergistic or neutral.

Materials and methods

Substratum and AMF material
Two parts of a substratum consisting of soil, sand and “Leca” in a 3:3:1 ratio were mixed with one part of a commercial available AMF inoculum (“Symbavit”, SYMbio-M; Czech Republic). Depending on the treatment, autoclaved (for the control and for the 3 *Trichoderma* treatments) or non-autoclaved fungal inoculum was used.

Fungal material
Three different biocontrol strains (*Trichoderma viride* strain RE 1-3-4, *T. harzianum* strain T39, *T. atroviride* strain P1) and the pathogen *F. oxysporum* f. sp. *lycopersici* strain 007 were used. The *Trichoderma* strains were grown on potato dextrose agar, *Fusarium oxysporum* was grown on Czapek Dox medium. Under sterile conditions fungal culture plates were flooded with sterilized water and the resulting spore suspension was filtered through three layers of filter paper to separate the conidia from the mycelium. The spore suspension was adjusted to the final concentration using a haemocytometer.
Experimental set-up
Four-week-old seedlings of *Solanum lycopersicum* L. cv. Moneymaker were used as plant material. Twenty plants were used per treatment (AMF, non-AMF, *Trichoderma viride* strain RE 1-3-4, *T. harzianum* strain T39, *T. atroviride* strain P1 and the 3 different *Trichoderma* strains together with AMF). *Trichoderma* strains were added as a spore suspension (2×10⁶ conidia/ml) at a volume of 5 ml per pot one week after potting. Plants were irrigated with a liquid fertiliser.

Collection of root exudates
After the inoculation period of 8 weeks the roots of the plants were washed and submerged in autoclaved distilled water for 6 h. Thereafter the root fresh weight was determined and the obtained exudates were adjusted with autoclaved distilled water to a concentration of 20 ml/g root fresh weight. Thereafter they were passed through 0.2 µm filters and stored at -20°C.

Root colonization
To visualize the AMF colonization, roots were cleared by boiling 4 min. in 10% KOH, rinsed three times with tap water and stained according to the method of Vierheilig et al. (1998) by boiling for 4 min in a 5% ink (Shaeffer; black)/household vinegar (= 5% acetic acid) solution. After staining, the percentage of root colonization was determined using the gridline intercept method (Giovannetti & Mosse, 1980).

Fungal growth assay
The fungal growth assay was carried out according to Broekaert et al. (1990) and Ludwig & Boller (1990). Aliquots of 175 µl of sterilized distilled water, Czapek Dox medium or root exudates were mixed with 35 µl of a microconidia suspension of *F. oxysporum* f. sp. *lycopersici* (1.0×10⁷ microconidia/ml) in sterile 96-well-culture plates and incubated at 24°C in the dark while shaking for 5 days. Fungal growth was assessed by an optical density measurement which was carried out every 24 h using a microtiterplate reader (Tecan, Spectra).

Results and discussion
Few data are available on the effect of tomato root exudates on the highly specialized tomato pathogen *Fol*. Steinkellner et al. (2005) reported that the microconidia germination of *Fol* is stimulated in presence of tomato root exudates. In a most recent report Scheffknecht et al. (2006) showed that in presence of root exudates from mycorrhizal tomato plants the microconidia germination of *Fol* is even higher than in presence of root exudates from non-mycorrhizal tomato plants indicating that mycorrhization alters the exudation pattern of plants. Our results confirm that the root exudation of tomato plants is altered through mycorrhization. However, we found that the fungal growth of *Fol* was negatively affected by the presence of root exudates from mycorrhizal tomato plants (Fig. 1), whereas microconidia germination was enhanced in presence of root exudates from mycorrhizal tomato plants (Scheffknecht et al., 2006). This indicates that the alterations in the exudation pattern through mycorrhization affect microconidia germination and fungal growth of *Fol* differently. To our knowledge little is known about possible alterations of root exudation in presence of *Trichoderma* spp. We found a clear indication that *Trichoderma* spp. alters the exudation pattern and that these alterations affect the fungal growth of *Fol* and thus are possibly involved in the bioprotectional effect of *Trichoderma* spp. towards *Fol*. However, the picture is not clear yet as depending on the tested *Trichoderma* strain we observed an inhibitory (*Trichoderma* strain T39 and RE) or a stimulatory effect (*Trichoderma* strain P1) on fungal growth of *Fol* (Fig. 1). Further studies are needed to elucidate the exact role of alterations of the root exudation by *Trichoderma* spp. in the bioprotectional effect against *Fol*. 
The inhibitory effect on fungal growth of *Fol* when the different *Trichoderma* strains were applied in combination with the AMF were similar as with AMF alone (Fig. 1), indicating that the alterations of the exudation pattern due to mycorrhization are more important than the effects caused by alteration of the root exudation by the *Trichoderma* strains. There was only one exception: Fungal growth in root exudates from the treatment *Trichoderma* strain T39 + AMF was even more reduced than in the AMF treatment. This could mean that *Trichoderma* strain T39 and AMF alter the root exudation in a similar way, both microorganisms enhancing the exudation of inhibitory compounds.

Fig. 2 shows that there is no significant difference (ANOVA, *P* > 0.05) in the root colonization between the treatments with AMF and AMF + *Trichoderma*, i.e. in this study *Trichoderma* spp. have no adverse effect on the root colonization of AMF. On the contrary in
the study of Green et al. (1999) the root colonization of cucumber by *Glomus intraradices* was significantly reduced in the presence of *T. harzianum*. McAllister et al. (1994 a,b) found for *Trichoderma koningii* that when inoculated before or at the same time as *Glomus mosseae*, mycorrhizal formation was reduced. However, when *T. koningii* was applied 2 weeks after *G. mosseae* no inhibition could be seen. In our study *Trichoderma* spp. was applied 1 week after the AMF. This might be the reason for no adverse effects on the degree of colonization. Furthermore, this interaction might be highly strain specific.

**Acknowledgement**

The authors are grateful to Gabriele Berg, Yigal Elad and Robert Mach and B.J. Cornelissen for kindly providing the fungal strains (RE 1-3-4, Th 39, P1 and Fol).

**References**


Changes in the root exudates of mycorrhizal tomato plants affecting microconidia germination of *Fusarium oxysporum* f. sp. *lycopersici* are not host specific

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Abstract: The effect of root exudates from plants colonized or non-colonized by the arbuscular mycorrhizal fungus *Glomus mosseae* on microconidia germination of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) was studied. Root exudates from the Fol-host tomato and root exudates from Fol non-host plants were tested. Root exudates from all tested plants stimulated microconidia germination. Mycorrhization increased the stimulatory effect exhibited by the root exudates from the Fol host tomato and from all non-host plants.

Key words: arbuscular mycorrhiza, biological control, Glomeromycota, plant diseases, plant-fungus interaction

Introduction

Root exudates of tomato plants stimulate the microconidia germination of the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Steinkellner et al. 2005). Root colonization by arbuscular mycorrhizal fungi (AMF) has been reported to alter the root exudation pattern of mycorrhizal plants (reviewed by Vierheilig, 2004) and most recently, Scheffknecht et al. (2006) demonstrated that the microconidia germination of Fol is even more stimulated in presence of root exudates from mycorrhizal tomato plants.

In the present work, we were interested whether microconidia germination-stimulating alterations of the root exudates through mycorrhization are limited to the Fol-host plant tomato or also occur in Fol non-host plants.

Materials and methods

*Biological material and growth conditions*

Tomato, tobacco, cucumber, onion, carrot, lettuce and papaya plants were used. Seeds were surface-sterilized in 50% commercial bleach for 5 min, rinsed three times in sterile distilled water and germinated in autoclaved (20 min; 121°C) perlite. Two weeks after seeding (except tobacco after 4 weeks), 5 plantlets per treatment (control or inoculated plants) were transferred into an autoclaved (20 min; 121°C) mixture of silicate sand, expanded clay and soil (1:1:1; by vol.), and were inoculated with *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe (BEG 12; European Bank for the Glomales). Plants were grown in a growth chamber (day/night cycle: 16 h; 23°C/8 h; 19°C; RH 50%; light intensity 430 µE/sec⁻¹ m⁻²) and watered with tap water every day.

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**Collection of root exudates**

Depending on plant species, the inoculation period with *G. mosseae* ranged between 2-5 weeks. After the inoculation period, mycorrhizal and non-mycorrhizal plants were harvested and roots of whole plants were washed and submerged in sterilized water for 24 h. The exudate obtained was adjusted with sterilized water (20 ml/g root fresh weight) and passed through millipore filters (0.2 µm). Thereafter the degree of root colonization and the root fresh weight were determined (for details see Scheffknecht et al., 2006).

**Germination assay**

Aliquots of 500 µl of root exudate were mixed with 100 µl of conidia suspension (*F. oxysporum* f. sp. *lycopersici*, 1.0×10^7 microconidia/ml water) in sterile 24-well-culture plates and incubated at 24°C in the dark while shaking. Microconidia germination was determined microscopically after 24 h by counting 200 conidia (for details see Steinkellner et al., 2005).

**Root colonization**

To determine AMF colonization, roots were cleared by boiling 4 min in 10% KOH, rinsed three times with tap water and stained according to the method of Vierheilig et al. (1998) by boiling for 4 min in a 5% ink (Shaeffer; black)/household vinegar (= 5% acetic acid) solution. After staining, the percentage of root colonization was determined using the gridline intercept method (Giovannetti & Mosse, 1980).

**Results and discussion**

We could show that not only root exudates of mycorrhizal tomato plants (Solanaceae) enhanced microconidia germination compared to root exudates of non-mycorrhizal plants, but also root exudates of mycorrhizal tobacco (Fig. 1), another Solanaceae, and root exudates of all other tested mycorrhizal Fol-nonhost plants (Fig. 2).
This indicates that changes in the root exudation of the Fol-host tomato through mycorrhization are not specific to the Fol-tomato interaction, which means that similar qualitative and/or quantitative alterations of the root exudates do also occur in Fol-nonhost plants of the same plant family (the Solanaceae) as tomato and in root exudates of mycorrhizal plants from other plant families. Studies are underway to identify these qualitative and/or quantitative alterations of the root exudates in mycorrhizal plants.

Figure 2. Effect of H2O, Czapek Dox (CzD) solution, root exudates from mycorrhizal (+M) and non-mycorrhizal (-M) plants from different plant families on the germination of microconidia of Fusarium oxysporum f. sp. lycopersici. Values of the H2O, CzD controls and the percentage of root colonization are given on top of exudate data. Data represent mean ± S.E. (n = 6).

Acknowledgements

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References


Biology and biological control of tomato powdery mildew  
(Oidium neolycopersici)

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Abstract: Tomato powdery mildew (Oidium neolycopersicum) has started to cause severe epidemics on tomato about 15 years ago. It mainly infects Solanaceae and Curcubitaceae plants. The symptoms on tomato plant include powdery white lesions on the upper side of leaves and on all other aerial plant parts except for on the fruits. In severe outbreaks, the lesions coalesce and the disease is debilitating resulting in fast death of leaves. It is extremely common in greenhouse tomatoes world-wide but increasing in importance on field-grown tomato crops. Suppression of the pathogen relies mainly on chemical fungicides. The fast nature of epidemic development results in many and frequent sprays. Our objectives were to study the disease biology and to find new friendly means of control of the disease including biocontrol agents in order to later structure an integrated control system that will result in reduction of chemical fungicides use and residues. O. neolycopersici conidia germination was highest at temperature 25°C, high relative humidity (RH) and low light intensity, whereas, conidia production is higher at 22°C, lower RH and higher light intensity (but fluctuating greenhouse temp result in higher number of conidia). Under field conditions, epidemics developed faster under conditions of high RH and moderate temperature. Accordingly, we simulated conditions for significant epidemic development on potted tomato plants subjected to artificial inoculation in experimental greenhouses. While testing various yeast and bacterium isolates, it was found that few of them reduced disease severity by up to 80% on potted plants under heavy disease pressure. However, disease suppression was better on upper leaves than on lower leaves. Disease severity was significantly higher on the lower leaves, thus control efficacy may be negatively related to the disease pressure and the time the plant organ was exposed to the pathogen inoculum and the biocontrol agent. The potential biocontrol agents did not affect the germination and germ tube elongation of the Oidium conidia. However, three of the micro-organisms reduced conidia formation on the thallus of O. neolycopersici on leaves.

Key words: biocontrol, life cycle

Introduction

Tomato powdery mildew caused by Oidium neolycopersicum has started to cause severe epidemics on tomato during the last 15 years (Jones et al., 2001). Heavy infections were first recorded in Western Europe and during the 1980s it was reported for the first time in North America (Mieslerová & Lebeda, 1999). It is a dangerous pathogen, which spread through temperate areas of the world. The host range of the pathogen is broad and it is reported to attack over 60 species in 13 plant families, particularly members of Solanaceae and Curcubitaceae. (Jones et al., 2001). Symptoms include powdery white lesions on the adaxial leaves surface and on all other aerial plant parts except on the fruits. The fruits are not directly affected but impaired photosynthesis and premature senescence reduces fruit size and nutritional quality leading to diminished yields (Mieslerov’a & Lebeda, 1999). In severe outbreaks, the lesions coalesce and the disease is debilitating resulting in fast death of leaves.
It is extremely common in greenhouse tomatoes worldwide but increasing in importance on field-grown tomato crops. Suppression of the pathogen relies mainly on chemical fungicides. The fast nature of epidemic development results in many and frequent sprays.

The objectives of this study was to study the disease biology and to find new friendly means of control of the disease including biocontrol agents, in order to later structure an integrated control system that will result in reduction of chemical fungicides use and residues.

**Material and methods**

**Conidia germination**
Experiments were done to establish the optimum conditions for conidia germination. Conidia were brushed from freshly conidiating leaf lesions on the surface of healthy leaf. Leaves were then incubated for 24 h under different temperature, RH and light intensity and then observed under light microscope.

**Biocontrol treatments**
Bacteria (B69, B71) and yeast (Y16, Y2, Y89, Y13) isolated from the canopy of wild and cultivated plants were grown in liquid culture (NB and YMDB, respectively), centrifuged, re-suspended and sprayed on tomato whole plants or single leaves at rate of 10^8/ml.

**Effect of BCAs on conidia germination**
Inoculated detached leaves were placed in a humidity chamber at 25±1°C and 3250 Lux for 24 h after being sprayed with the BCAs (30 µL of 10^8/ml). Germination was observed and evaluated under light microscope. Percent germination was registered by counting the number of conidia with germ-tube exceeding the length of conidium (12 replicate, 100 conidia each). The untreated control treatment consisted of water spray without a BCA and no spray at all.

**Effect of BCAs on appressoria formation**
Inoculated detached leaves were placed in in a humidity chamber as described above. The BCAs were applied to leaves 12 h after infection and appearance of appressoria was observed 24 h later. Observation of appressoria formation was conducted under light microscope. The percentage of appressoria formation was calculated by counting the number of appressoria out of 100 conidia. (12 replicates). The untreated control treatment consisted of water spray without a BCA and no spray at all.

**Effect of BCAs on conidiation**
BCAs were sprayed at the rate of 10^8/ml twice a week on whole tomato plants. The first spay was carried out 3 hours after inoculation with a suspension containing 5*10^4 conidia/ml. The density of conidia formed was examined every 5 day by shaking each treated leaf with 1 ml of H2O. Conidia were counted with a haemocytometer under light microscope. (12 replicate). Values of conidia accumulation per leaf area were plotted in a graph and area under progress curve (AUPC) was calculated for presentation.

**Effect of BCAs on disease severity**
BCAs were sprayed at the rate of 10^8/ml twice a week on whole tomato plants. The first spay was 3 days before inoculation with conidia suspension containing 5*10^4 conidia/ml. Disease symptoms appeared on leaves 7-10 days after inoculation. Severity of disease was evaluated according to the proportion of leaf area covered by typical disease symptoms. According to a scale between 0-100% where 0=healthy (no visible symptoms) and 100=entire leaf covered by symptoms. Disease severity was evaluated every 5 days from inoculation, plotted in a graph and area under disease progress curve was calculated for presentation. There were 6 replicates per each treatment.
Results

Conidia germination
Experiments were done to establish the optimum conditions for conidia germination. Conidia were brushed from freshly conidiating leaf lesions on the surface of healthy leaf. The conidia germination of *O. neolycopersici* was highest at temperature of 25ºC, high RH and low light intensity. (Result not shown). The effect of BCAs on conidia germination was tested after incubation for 24 h on leaves. Only one yeast and one bacterium isolate slightly inhibited germination and germ tube elongation of the conidia, respectively (Result not shown).

Effect on appressoria formation, conidiation and disease severity
All the six micro-organisms significantly reduced the appressoria formation (23-66% reduction) compare with the control and the water treatment (Table 1). Four microorganisms (2 yeast and 2 bacteria) significantly reduced conidia production on infected leaves (55-66% reduction) as compared with the control and the water treatment. (Table 1) All the six microorganisms significantly reduced disease severity on whole plants (37-82% reduction) (Table 1).

Table 1. Effect of six BCAs on *O. neolycopersici* appressoria formation and conidiation on leaves and disease severity on whole tomato plants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Appressoria formation</th>
<th>Conidia formation</th>
<th>Disease severity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germinated conidia (%)</td>
<td>AUPC conidia/cm² leaf×days</td>
<td>AUDPC (% leaf coverage×days)</td>
</tr>
<tr>
<td>B69</td>
<td>10.8</td>
<td>16.0</td>
<td>82.2</td>
</tr>
<tr>
<td>B71</td>
<td>16.5</td>
<td>13.6</td>
<td>65.4</td>
</tr>
<tr>
<td>Y16</td>
<td>8.5</td>
<td>10.8</td>
<td>77.3</td>
</tr>
<tr>
<td>Y89</td>
<td>19.5</td>
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<tr>
<td>Y2</td>
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<td>63.8</td>
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<tr>
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<td>15.1</td>
<td>23.3</td>
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<tr>
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<td>nt</td>
</tr>
<tr>
<td>Control</td>
<td>25.4</td>
<td>32.1</td>
<td>136.1</td>
</tr>
</tbody>
</table>

Nt= not tested.

Conclusions

Powdery mildew causes severe damage on greenhouse tomato has been reported worldwide. The main goal of this research was to study the disease biology and to find new friendly means of control of the disease including biocontrol agents in order to later structure an integrated control system that will result in reduction of chemical fungicides use and residues.

In this study the tested micro-organisms affected appressoria formation but not germination. Some of them even reduced conidia production of the leaves. It may be concluded that a mode of action related to later stages of infection and pathogen development or host resistance might be involved.

Our main goal in the future is to test selected BCAs under field conditions as stand-alone or in combination with other means of control and thus to reduce the number of chemical sprays.
References


Dynamics of microbial communities associated with genetically modified sugarcane and the biocontrol potential of endophytic bacteria against *Fusarium moniliforme* Kleiner

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Abstract: The microbial community of sugarcane was accessed by using isolation and DGGE technique. In the field experiment were considered 3 treatments, following (1) sugarcane SP80-1842 (conventional) weeded; (2) sugarcane IMI-1 imazapyr resistant weeded; and (3) sugarcane IMI-1 imazapyr resistant managed with herbicide. The microorganisms were isolated after superficial disinfection of leaves and roots of sugarcane plants, also it were isolated fungi from rhizosphere. The genetic variability was evaluated by rDNA sequencing. The non-cultivated community of fungi was accessed through DGGE. It was not observed any relation between observed microorganisms and treatments, concluding that in the evaluated conditions neither transgeny nor management affect the microbial population.

Several *Fusarium* spp. were isolated from sugarcane and the molecular characterization of this group was performed by using RAPD. It was observed pathogenic and non-pathogenic *F. moniliforme* associated to sugarcane. 170 endophytic bacteria, isolated from the same experiment, are being evaluated against *F. moniliforme*, causal agent of pokkah boeng disease in sugarcane. The endophytic bacteria were characterized by BOX-PCR, and tested *in vitro* by pairing with *F. moniliforme* in PDA media. It was observed a number of bacteria with biocontrol potential and further assays are needed to understand the mechanism of this antagonistic interaction. Financial Support: FAPESP and CAPES.
Understanding naturally occurring antagonists
Interest of cultural practices to manage soilborne diseases

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Abstract: Soilborne diseases are caused by pathogenic agents constituting single populations among complex communities of microorganisms. These populations are interacting and regulating their densities and activities, also modulated by the a-biotic environment. Cultural practices such as the application of organic amendments, rotation of crops, intercropping, bio-disinfestation, solarisation, and tillage practices influence the balance between functional groups in soils. Some of these cultural practices, alone or in combination have the capacity to enhance disease suppression but their efficacy depends on the resident microflora of the soil and on the specific ecological requirements of the pathogens. More detailed investigation of the biologically complex system and improvements of the presently available methods are necessary to improve practices in order to manage this microbial potential in an economically and feasible manner.

Key words: soil suppressiveness, soil inoculum potential, soilborne microbial communities, crop rotations, organic amendments

Introduction

The soil is often considered as the milieu providing support for plant roots, and also water and nutrients for plant growth. But it is also considered as a hostile environment harbouring plant pathogenic nematodes, bacteria and fungi. The most common attitude is to try to eliminate the plant pathogenic organisms by biocidal treatments. These practices include high pressure water steam but also wide spectrum chemicals. However, the specific eradication of the pathogen is rarely reached. Moreover, fumigants are dangerous for man and the environment. Another approach to control soil-borne plant diseases consists in studying the plant-pathogen interactions at the cellular and molecular level to create new resistant cultivars or to develop new plant protection products based on elicitation of plant defence reactions. This field of research only focuses on plant pathogen interactions, not taking into account the environment where they take place.

Indeed, the pathogens are not freely interacting with the plant; they are included in the soil matrix and thus can not escape to the soil environment. Both their inoculum density and infectious capacities are controlled by the soil. Evidence of these interactions is given by the existence of soils that suppress diseases. In suppressive soils disease incidence or severity remains low in spite of the presence of the pathogen, a susceptible host plant and climatic conditions favourable for disease development. These suppressive soils provide examples of biotic and abiotic factors affecting the pathogen, the plant or their interactions. In other words, each soil is putatively suppressive providing the achievement of the relevant combination of these biotic and abiotic factors (Alabouvette et al., 1996; Steinberg et al., in press).

Therefore, many studies have been devoted to the understanding of soil suppressiveness in order to use suppressive mechanisms in biological control strategies. Since antagonistic
micro-organisms play a role in soil suppressiveness in many cases, the main approach was to identify effective antagonists in soil for the three last decades and to develop them as biological control agents for both aerial and soilborne pathogens. But in order to control soilborne diseases, one must admit that this strategy has not been as successful as expected. Indeed, even if the soil harbours effective antagonists, soil suppressiveness is due to an association of mechanisms and micro-organisms, and a single antagonist is never as efficient as the suppressive soil itself. Thus an alternative approach, more ecological, consists in enhancing natural suppressiveness that exists in every soil. Some cultural practices might modify the microbial balance in a way that soil inoculum potential will be decreased, and/or the soil suppressiveness increased. We will present in this mini review some aspects to implement this alternative approach.

Role of agricultural practices on disease suppression

In a recent review, Garbeva et al. (2004) underlined the major repercussion of plant type, soil type and soil management on the microbial communities and on the soil suppressiveness to soilborne diseases. Mazzola (2004) and Janvier et al. (2006, 2007) have further detailed the management of soil microbial community structure for disease suppression. Agricultural practices should at the least preserve microbial populations favourable to the phytosanitary quality and if possible even favour them. An improved identification of those microorganisms that contribute in a favourable (or unfavourable) way to this quality should help growers make relevant decisions when choosing agricultural practices.

Disease suppressive soils were shown to develop through crop rotation (Bailey et al., 2001), intercropping (Schneider et al., 2003), residue destruction (Baird et al., 2003), organic amendments (Bailey & Lazarovitz, 2003) tillage management practices (Pankhurst et al., 2002) and combination of those regimes (Hagn et al., 2003).

Various studies have clearly indicated that the microflora associated with roots differs according to the plant species and even cultivar. From these observations, the possibility to manage the microbial communities associated with the roots through the plant cultivation has been proposed. A famous example is the demonstration of the possibility to increase the density of 2,4-DAPG-producing fluorescent pseudomonads in the rhizosphere of wheat to a threshold high enough to suppress take-all by the monoculture of this plant species in the presence of Gaeummanomyces graminis var. tritici (Raaijmakers & Weller, 2001). Mazzola (2004) reported natural suppressiveness to apple replant disease caused by Rhizoctonia in soil that had a wheat monoculture prior to apple orchard establishment. Based on this observation, Mazzola & Gu (2002) have evaluated the possibility to use wheat cover cropping as a mean to re-establish a disease suppressive microbial community in orchard planted after a wheat monoculture. The efficacy of this agricultural practice was shown to differ according to the wheat cultivars and to their ability to select specific populations of fluorescent pseudomonads (Mazzola, 2002). Depending on the permanence of the rhizosphere effect, the populations selected by the plant will just improve the health of the current crop or may also improve that of the forthcoming one. As example, this permanence was shown to be inexistent in the case of the take-all decline, since the interruption of this crop monoculture with an alternative crop reduced or abolished soil suppressiveness toward the corresponding pathogen (Cook, 1981).

Although organic amendments, including animal and green manures, were primarily the principal method of fertilization of the soils, they rapidly revealed some potential for the control of soil-borne diseases. The use of organic amendments including composts has been extensively reviewed by many authors (Hoïtink & Boehm, 1999; Noble & Coventry, 2005; Zinati, 2005). These studies generally reveal that one type of compost, when used in
controlled conditions, i.e. container based productions, can successively suppress the disease caused by a single or a few pathogens. Actually, in field, a host plant has to face infections by multiple pathogens. Furthermore, compost is known as a product that varies considerably in chemical, physical and biotic composition, and, consequently, also in ability to suppress soil-borne diseases. So, one may select for a compost which is highly suppressive to one disease while it has no effect against other important root diseases. The suppressive ability of a wide range of composts towards a wide range of plant pathogens has been recently evaluated (Termorshuizen et al., 2006). None of the 18 composts tested can be considered as the universal disease suppressor, but none of the pathogens can escape the suppressive effect of at least one of the composts. It is noteworthy that composts showed disease suppression and stimulation respectively.

Application of compost has in general a positive or no effect on disease suppression, and only rarely a disease stimulating effect. Propagules of plant pathogens such as *F. oxysporum* are suppressed through the general suppression phenomenon based on microbial competition for energy and it involves the total soil microflora (Cook & Baker, 1983). This mechanism is activated by organic amendments. To control pathogens such as *R. solani* which are less sensitive to general competition, a narrow group of specific antagonistic microorganisms (mycoparasites for instance) are required. Such microorganisms may already pre-exist in the soil and need to be stimulated by organic amendments. They can also exist in the compost and thus will be brought to the soil through the compost amendments.

The absence of a specific suppression may explain the neutral or disease stimulating effect of organic amendments towards competition independent pathogens while the same organic amendments are efficient towards competition sensitive pathogens. Control of soilborne plant pathogens was improved using the incorporation of fresh organic matter in combination with a plastic cover and solar heating (Blok et al., 2000). Although modifications of functional diversity as well as of genotypic diversity of microbial communities following organic amendments have been reported (Edel-Hermann et al., 2004; Pérez-Piquerez et al., 2006), it has been very difficult to reveal the particular stimulation of specific indigenous populations exerting an antagonistic activity towards the pathogens.

Beside the effect of organic amendments on beneficial microflora through mechanisms of general and/or specific suppressiveness, introduction of plant residues may also affect the pathogen. This is the case for Brassicaceae that produce glucosinolates, a class of organic anions, which may represent a viable source of allelochemic products to control various soil borne pathogens (Kirkegaard & Sarwar, 1998). Toxicity is not attributed to glucosinolates but to products such as isothiocyanates, organic cyanides or ionic thiocyanates resulting from their enzymatic degradation achieved by a group of similar-acting enzymes called myrosinase. Based of this property, Brassicaceae may be cultivated as intermediate crop or enter the rotation, and are buried into the soil as green manure. The hydrolysis products have a broad biocidal activity towards nematodes, insects and fungi as well as putative phytotoxic effects. They act either as selective fungicides or as fungistatic compounds limiting the development and activity of fungal populations, some of them being putative pathogenic agents for the forthcoming crop (Sarwar et al., 1998). The suppressive effect towards diseases caused by pathogenic fungal populations is attributed to a direct reduction of the pathogen inoculum density but it could be partly attributed to changes in the structure of the indigenous and beneficial microflora. However, the allelopathic effect may negatively alter the indigenous microflora leading to subsequent conduciveness of the soil to other diseases. Therefore, it is necessary to evaluate the incidence and the persistence of the glucosinolates effect on both the pathogenic populations and the microbial communities. Similarly the
resilience of the microbial communities and the putative come back of the pathogens as the emergence of previously undetected pathogenic populations need to be evaluated.

Another example of organic amendment affecting directly the pathogens may be illustrated with the control of Allium white rot due to Sclerotium cepivorum partly achieved by incorporating composted onion wastes into the soil. Roots of Allium species release alk(en)yl cysteine sulfoxides which favour germination of the pathogenic spores. Properly composted, onion wastes including these compounds will trigger the germination of dormant sclerotia which in the absence of the roots of the host plant will lead to a decreased density of primary inoculum (Coventry et al., 2002). However, in some cases plant debris may preserve also the pathogens, increasing thus primary inocula of M. phaseolina causing charcoal rot in soybean (Baird et al., 2003), Fusarium sp. causing root and crown rot, stalk rot and ear rot on maize (Cotten & Munkwold, 1998), L. maculans causing stem canker or blackleg on oilseed rape (Schneider et al., 2003), R. solani causing crown and root rot on sugar beet (Guillemaut, 2003). Therefore, attention should be paid to residue management by burial through tillage practices or promotion of rapid decomposition (Toresani et al., 1998).

It is difficult to assess the role of tillage on disease suppression as its evaluation is often combined with the effects of other agricultural (Bailey & Lazarovits, 2003). Therefore tillage appears as giving conflicting effects on disease suppression. Conventional tillage results in considerable disturbance of the soil but removes residue and thus lowers the potential for diseases by displacing the pathogens from their preferred niche to deep layers of the soil. Tillage also disrupts hyphae altering for instance the ability to survive of R. solani (Bailey & Lazarovits, 2003). Reduce tillage systems change the availability of nutrients in the soil increasing microbial biomass, microbial activity and subsequent competition effects. Total soil nitrogen, organic matter and denitrification processes are increased but mineralization and nitrification processes are reduced. Soil inoculum potential and disease incidence might be differently altered according to pathogens. Reduced tillage can favour pathogens by protecting the pathogen's refuge in the residue from microbial degradation, lowering soil temperature, increasing soil moisture, and leaving soil undisturbed (Bockus & Shroyer, 1998). Indeed, the impact of tillage practices may depend on a specific pathogen-soil-crop-environment interaction, environment being sometimes the most important factor limiting the severity of disease regardless of tillage or crop rotation practices (Bailey et al., 2000).

Conclusion

The few examples mentioned here show how complex are the interactions between soil characteristics, microbiota, pathosystems and disease suppression. All the microbial populations including bacteria, fungi, protozoa, nematodes, enchytreids, earthworms … are involved in the soil functioning but with the constraints of the environment. Therefore, to increase soil suppressiveness, we need to better understand the effects of management practices on the diverse components of soil health, and therefore to determine when and what kind of management is necessary to help farmers make the right decisions. Although the mechanisms responsible for soil suppressiveness to diseases are not yet elucidated, many examples clearly show that such a property might be acquired by the soils thanks to agricultural practices. Developing disease suppressive soils by agricultural practices will probably not provide immediate return compared to the use of fumigants or pesticides, but the benefits accumulate across successive years and improve soil health and structure. Producers should not rely exclusively on a single management practice but a combination of practices should be integrated to develop a consistent long term strategy for disease management that is suited to their production system and location.
References


Organic matter-mediated cocoyam root suppression in natural and field systems in Cameroon

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Abstract: The root rot disease caused by *Pythium myriotylum*, a soil-borne pathogenic oomycete, is the major limiting factor to increased production of cocoyam (*Xanthosoma sagittifolium*), an economical important food crop in the tropical and sub-tropical region. Suppressive soils to cocoyam root rot in Cameroon have been studied since 2001 in view of identifying factors responsible for the disease suppression. Greenhouse plant assays using artificially infected natural, heated and microbially-recolonised heated suppressive soils, and a comparative analysis of properties of suppressive and conducive soils were used to identify soil factors that mediate the disease suppression. Physicochemical variables such as high content of soil organic matter, N, cations, and biological variables including high population densities of heterotrophic bacteria, *Pseudomonas* spp., Actinomycetes, and *Trichoderma* spp. were found to be strongly associated with soil suppressiveness. Three compost types previously screened for suppressiveness to *P. myriotylum*, were used in attempts to generate suppressiveness in disease conducive soils. Compost amendments at the rate of 20 t ha⁻¹ raised the soil organic carbon content to an average up to 2% (about one quarter of that in actual suppressive soils; 8.12 to 8.72%). This resulted in significant disease reduction and increased tuber yield in three experimental plots (Ekona, Matomb and Akonolinga) differing in their cropping histories. But the disease control and the tuber yield were satisfactory only at Ekona (68.3 to 70.4% disease reduction and 71.5 to 75.84% increase tuber yield) where the inoculum pressure was relatively lower (3.7×10² cfu/g soil), and unsatisfactory at Matomb (20.5 to 30.1% disease reduction and 0 to 25.8% increase tuber yield) and Akonolinga (10.2 to 24.4% disease reduction and 34 to 59% increase tuber yield) where the inoculum pressure was relatively higher, (5.27×10² and 5.43×10² cfu/g soil), respectively. Results suggest reduction of *Pythium* population in soil through crop rotation prior to applying composts.
Effect of calcium lignosulphonate on sclerotia of *Sclerotinia sclerotiorum* in organic substrates

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**Abstract:** Our purpose was to evaluate the effect of calcium lignosulphonate (CaLs) addition (3% v:v) to three organic substrates for ornamental plants: peat + coconut fibre (PC), municipal solid wastes + peat + pumice (MCP), and green compost + peat + pumice (GCP) on sclerotia of *Sclerotinia sclerotiorum*. Sterile sand was used as non-organic control. Sclerotia were buried into each substrate enriched or not with CaLs immediately after the addition of CaLs or sterile water. After 30 days, sclerotia were removed, surface sterilised and plated on agar medium to verify viability and colonisation of sclerotia by fungi naturally present in the substrate. CaLs significantly reduced the sclerotial germination when added to PC, whereas no effect was observed when it was added to GCP or sterile sand. The biocontrol effect of CaLs against sclerotia buried in MPC was transient. The enrichment of organic products with CaLs stimulated the colonisation of sclerotia by *Trichoderma*, *Mucor* and *Fusarium oxysporum*.

**Key words:** green compost, municipal solid wastes compost, organic amendment, sclerotial viability

**Introduction**

*Sclerotinia* spp. are polyphagous plant parasites with an extremely voluminous phytopathological literature. They survive as sclerotia in soil or in plant tissues. Survival is adversely affected by microbial activities (Domsch et al., 1993).

Lignosulphonates (Ls) are low cost by products of the acid sulfite pulping process; millions of tons are produced yearly and most is incinerated. They have a lignin-like structure, containing mono (20%) and polysaccharides (5%). In previous tests, mature spent mushroom compost (MSMC) showed highly suppressive ability against the disease caused by *Fusarium oxysporum* f. sp. *melonis* on melon plants; the addition of Calcium Ls (CaLs) and *Trichoderma atroviride* separately did not affect the biocontrol ability of MSMC, whereas the enrichment with both drastically enhanced the compost suppressiveness (Montanari et al., 2004). The increase of NH$_4$N level and β-glucosidase activity observed after the fortification with CaLs, could create in the mushroom compost an environment suitable for *Trichoderma* (Montanari et al., 2004). Lazarovitis (2001) and Soltani et al. (2002) showed that Ammonium Ls (ALs) added to field soil significantly reduced potato scab and Verticillium wilt severity on potato plants. Lazarovitis (2001) observed that in *in vitro* tests, ALs treatment reduced *Streptomyces* numbers suggesting that some direct toxicity against the pathogen might be involved in the biocontrol activity of ALs (Lazarovitis, 2000).

The aim of this study was to investigate the effect of CaLs on sclerotia of *Sclerotinia sclerotiorum* (Lib.) De Bary buried into organic substrates for ornamental plants characterised by different origin, composition and age.
Material and methods

Organic products
The following organic products were used: peat (88%) + coconut fibre (12%) (PC; Stender s.r.l., Italy), municipal solid wastes compost (25%) + peat (15%) + pumice (60%) (MCP; Nuova Geovis s.r.l; Italy), green compost (25%) + peat moss (15%) + pumice (60%) (GCP; Nuova Geovis s.r.l; Italy). Sterile sand (S) was used as non-organic control. All products were maintained at 4°C in the dark until the beginning of experiments.

Sclerotia assays
– First experiment: This experiment was conducted in January, 2005 using PC and MCP. Each product was distributed into plastic containers (70 ml) closed by plastic film; ten replicates were made for each product. Ten sclerotia of *S. sclerotiorum* isolated from geranium plants and cultured on sterile wheat grain (Clarkson et al., 2003), were randomly buried into each container. Immediately before the inoculation with sclerotia, liquid CaLs (LS, Bretax C®, Cartiere Burgo, Tolmezzo, UD, Italy) was added (3% v/v) to the half of containers, sterile water was added to the other half of containers. All substrates were maintained at 60% water content, 20±2°C, in the dark. After 30 days, sclerotia were removed from the substrate; washed thoroughly under running water, surface-sterilised with sodium hypochlorite (1%) for 2 min, then rinsed in sterile distilled water, and plated on mPDA (Postma et al., 2003) to verify the germination and colonisation of sclerotia by other fungi.

– Second experiment: The experiment was carried out six months later, using the same organic products used in the first experiment. GCP was used for the first time. At the beginning of the experiment, all containers were inoculated with sclerotia, and a half of containers was enriched with CaLs, as reported above.

– Third experiment: Experiment was repeated nine months after the first, using PC, MCP, GCP.

All data are presented as untransformed, but, before analysis of variance, they were arc sin transformed. Data were subjected to multifactor ANOVA (two factors: product and CaLs). Data of the same product with and without CaLs were compared by Student *t* test (Statgraphics Plus, 1996).

Results

The results of germination assays are reported in Table 1. ANOVA analysis showed that both factors, product and CaLs, and their interaction are significant at *P*=0.05 in all experiments. All sclerotia removed from sand substrate (S) germinated on mPDA, independently of CaLs addition; whereas the enrichment of PC with CaLs significantly reduced the number of germinating sclerotia in all experiments. The number of germinating sclerotia removed from MCP + CaLs was significantly lower than that of sclerotia recovered from MPC without CaLs in the first exp., whereas no differences were found in the following experiments. The viability of sclerotia removed from GCP + CaLs was similar to that of sclerotia from GCP alone in all experiments.

Numbers (%) of sclerotia colonised by fungi naturally present in the products are reported in Table 2. In particular, *Trichoderma* spp. (Tr), *Mucor* sp. (Mu), and *Fusarium oxysporum* (Fo) were the most common colonizers of sclerotia of *Sclerotinia*. *Trichoderma* was isolated from sclerotia buried in PC + CaLs in all experiments, whereas this fungus was not isolated from sclerotia buried in PC without CaLs. In MCP + CaLs a significant increase of number of sclerotia colonised by *Mucor* in the first exp. and by *F. oxysporum* in the second exp. compared to that of MCP alone was observed. In GCP no significant presence of fungal
colonizers was observed after CaLs treatment. No fungi were isolated from sclerotia buried in S with and without CaLs.

Table 1. Germinating sclerotia (%) of Sclerotinia sclerotiorum 30 days after burial in organic products enriched or not with CaLs (3%; v:v)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>First exp.</th>
<th>Second exp.</th>
<th>Third exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile sand</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
</tr>
<tr>
<td>Sterile sand + CaLs</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
</tr>
<tr>
<td>Peat + coconut fibre</td>
<td>90 a</td>
<td>100 a</td>
<td>100 a</td>
</tr>
<tr>
<td>Peat + coconut fibre + CaLs</td>
<td>35 b</td>
<td>59 b</td>
<td>35 b</td>
</tr>
<tr>
<td>Municipal compost + peat + pumice</td>
<td>45 a</td>
<td>89 a</td>
<td>100 a</td>
</tr>
<tr>
<td>Municipal compost + peat + pumice + CaLs</td>
<td>10 b</td>
<td>67 a</td>
<td>100 a</td>
</tr>
<tr>
<td>Green compost + peat + pumice</td>
<td>90 a</td>
<td>100 a</td>
<td></td>
</tr>
<tr>
<td>Green compost + peat + pumice + CaLs</td>
<td>84 a</td>
<td>95 a</td>
<td></td>
</tr>
</tbody>
</table>

Data of the same product with and without CaLs followed by the same letter are not significantly different at $P \leq 0.05$ according to the Student $t$ test.

Table 2. Sclerotia (%) of Sclerotinia sclerotiorum colonised by Trichoderma spp. (Tr), Mucor sp. (Mu), and Fusarium oxysporum (Fo) 30 days after their burial into the organic products enriched or not with CaLs (3%; v:v)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>First exp.</th>
<th>Second exp.</th>
<th>Third exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile sand</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>Sterile sand + CaLs</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>Peat + coconut fibre</td>
<td>0b</td>
<td>0a</td>
<td>0b</td>
</tr>
<tr>
<td>Peat + coconut fibre + CaLs</td>
<td>100a</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>Municipal compost + peat + pumice</td>
<td>0a</td>
<td>0b 70a</td>
<td>0a</td>
</tr>
<tr>
<td>Municipal compost + peat + pumice + CaLs</td>
<td>0a</td>
<td>55a 100a</td>
<td>0a</td>
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<tr>
<td>Green compost + peat + pumice</td>
<td>5a</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>Green compost + peat + pumice + CaLs</td>
<td>11a</td>
<td>5a  5a</td>
<td>0a</td>
</tr>
</tbody>
</table>

Data of the same product and the same fungus with and without CaLs followed by the same letter are not significantly different at $P \leq 0.05$ according to the Student $t$ test.

Discussion

Our results show that the addition of CaLs to PC was able to significantly reduce the sclerotial germination independently of the age of product. On the contrary, the fortification of S and GCP with CaLs did not show any effect on sclerotia; whereas the reduction of sclerotial viability in MCP + CaLs was dependent on the age of the product. This seems to indicate that the reduction of sclerotial viability by CaLs cannot be attributed to a direct toxicity of CaLs against the pathogen, as suggested by Lazarovitis (2001). Under the experimental conditions considered, the biocontrol effect of CaLs against sclerotia of Sclerotinia seems related to microbial composition of the product, which changes in relation to origin and age of product. The addition of CaLs to organic products might stimulate indigenous fungal populations, comprising mycoparasites, presumably in response to increased nutrient availability.
References


Endophytes for the biological control of fungal tree diseases

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Abstract: Endophytic microorganism-plant relationships range from the symbiotic to the pathogenic. Pathogen-plant interactions have been intensively investigated in an effort to study the mechanisms of pathogenesis and the basis of host-parasite relations. On the other hand, plants’ interactions with endophytic, non-pathogenic microorganisms have received much less attention and are still poorly understood. Some of these non-pathogenic endophytes are beneficial to their host plants. Some endophytes may protect their hosts by secreting antibiotics which inhibit the abilities of pathogens to develop in the plant and cause disease. These beneficial endophytes are the target of this study. We are looking for endophytes that have the potential to serve as biological control agents against fungal pathogens of trees. This work describes the processes involved in the isolation of biologically active endophytes from citrus trees and the introduction of a unique fungus, Muscodor albus, as a potential biocontrol agent for use against some tree pathogens, including Phoma tracheiphila.

Key words: citrus, Muscodor albus, Phoma tracheiphila

Introduction

The associations between endophytic fungi and plants are ancient and diverse. These relations span from the mutualistic to the antagonistic, involving symbiotic microorganisms at one extreme and pathogenic microorganisms at the other. Endophytic fungi influence the diversity of the microbial community in the tree. Yet, very few of these nonpathogenic endophyte-tree relationships have been studied, with most research focusing on plant pathogens and mycorrhizal fungi (Arnold et al., 2003). There are examples of endophytic microorganism-plant relationships that provide specific benefits to host plants, including systemic resistance against pathogens, improved tolerance to heavy metals, increased drought resistance, reduced herbivory, and generally enhanced growth (Arnold et al., 2003; Bacon & White, 2000; Baraka et al., 2002; Chen et al., 1995; Joost, 1995; Narisawa et al., 2002; Paterson et al., 1995; Rubini et al., 2005). The control of systemic pathogens in trees is a very complex issue. The involvement of woody tissue, large plants and long life cycles makes it difficult to successfully control systemic fungal diseases with chemicals and agromechanical methods.

The idea of incorporating endophytes’ ability to develop systemically in trees and their ability to produce and secret antibiotics into a program for controlling systemic pathogens of trees is certainly logical. However, many obstacles arise in the implementation of this idea. One problem is that of host-pathogen specificity. Plant pathogens are generally specific to a single or few hosts; in most cases, individual pathogens are restricted to attacking and causing disease in specific hosts. Very little is known about the host specificity of other, nonpathogenic endophytes. In some cases, certain endophytic fungi have been found in several different hosts (Bayman et al., 1998). In other cases, the specificity of the host-endophyte relationship is not yet clear. One way to overcome this obstacle of specificity involves identifying active, endophytic microorganisms in plants related to the target plants.
A healthy tree in a diseased orchard may be explained in many ways. One explanation is the possible existence of an endophytic microorganism that helps the plant overcome the disease. In this case, finding, characterizing, studying and implementing this factor into other trees of the same species may provide a means for controlling the pathogen in other, similar orchards.

A second approach relies on the fact that the isolation of an endophyte from a number of different plant species indicates that the endophyte is not restricted to a specific host. The development of efficient methods for introducing the beneficial endophyte, monitoring its activity, if any, and ensuring its survival in the new host are all significant challenges. One example of a promising endophyte that may serve as a biological control agent for many woody plant diseases is *Muscodor albus*. This endophytic fungus was first isolated from a cinnamon tree (*Cinnamomum zeylanicum*) from Honduras (Woropong et al., 2001). Since its initial isolation, at least 14 new isolates have been isolated from different plants from different locations around the world (Daisy et al., 2002a; Daisy et al., 2002b; Ezra et al., 2004; Woropong et al., 2001). This fungus is unique in its ability to emit gases which are lethal to other fungi and bacteria. It also secretes soluble compounds which are active against some plant pathogens (Ezra et al., 2004; Ezra & Strobel, 2003; Woropong et al., 2001). This work presents endophytic bacteria and fungi isolated from Israeli citrus trees with the potential to control *Phoma tracheiphila*, the Mal secco disease agent of citrus. The potential of *M. albus* to serve as a biological control agent against the citrus disease caused by, *P. tracheiphila* was a particular focus of this work.

**Materials and methods**

*Isolation and evaluation of endophytes*

Woody tissue samples were collected from citrus orchards all around Israel. Plants exhibiting extreme resistance to disease were sampled. Healthy trees in dying orchards, trees exhibiting durability in abandoned orchards or trees growing in areas where their chances of survival were not high were the target of our search. Plant samples were brought to the laboratory and surface sterilized by burning with alcohol. Sterilized pieces of wood were cut into 0.5×0.5 cm chips and placed on potato dextrose agar (PDA) plates. The subsequent growth of microorganisms was monitored. Microorganisms growing out of the plant tissue were collected and transferred to fresh PDA plates. The antifungal activity of all isolated microorganisms was monitored. Microorganisms growing out of the plant tissue were collected and transferred to fresh PDA plates. The antifungal activity of all isolated microorganisms was tested. Each isolate sample was placed in the center of a PDA plate, where it was cultured for a few days. After this initial establishment period, test fungi, the plant pathogens *Pythium* spp., *P. tracheiphila*, *Colletotrichum* spp., *Rhizoctonia* spp., and others, were placed around the edges of the plate, four for each plate. The growth of the test fungi in the presence of the field isolates was monitored daily. Endophytes exhibiting biological activity were grown for 5 to 14 days on potato dextrose broth (PDB) and filtered through a 0.45 µm membrane. By growing the test fungi on changing concentrations of this filtrate, we could determine the lowest concentration capable of inhibiting the growth of the fungus. This test provided us with a way to evaluate the endophytes’ potency against other plant pathogens.

*Identification of endophytes*

Endophytic fungi showing significant biological activity were identified through microscopic observation, focusing on fungal structures and spore characteristics. The identities of all fungi were confirmed by rDNA sequencing. PCR amplification of the ITS1, 5.8S, ITS2 region of the rDNA was conducted using specific primers (Table 1). Endophytic bacteria were identified by PCR amplification and sequencing of the 16S rDNA, using specific primers.
Sequences were compared to those in GenBank using the INCB blast software on the NCBI web site (http://www.ncbi.nlm.nih.gov/).

Table 1. Primers used for PCR amplification in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Product length (bp)</th>
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<th>Target</th>
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<tr>
<td>ITS1</td>
<td>5’ TCCGTAGGTGAACCTGCGG 3’</td>
<td>~500</td>
<td>White et al., 1990</td>
<td>rDNA ITS1-5.8S-ITS2</td>
</tr>
<tr>
<td>ITS4</td>
<td>5’ TCCTCCGCTTATGGATATGC 3’</td>
<td>~500</td>
<td>White et al., 1990</td>
<td>rDNA ITS1-5.8S-ITS2</td>
</tr>
<tr>
<td>BSF 8/20</td>
<td>5’ AGAGTTTTGATCCTGGCTCAG 3’</td>
<td>~1000</td>
<td>Wilmotte et al., 1993</td>
<td>16S rDNA</td>
</tr>
<tr>
<td>BSR</td>
<td>5’ GGGTTGCGCTGTCGTTRC3’</td>
<td>~1000</td>
<td>Wilmotte et al., 1993</td>
<td>16S rDNA</td>
</tr>
<tr>
<td>MalbusF</td>
<td>5’ GGGAGGCTACCCTATAGGGGA TAC 3’</td>
<td>~500</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>MalbusR</td>
<td>5’ CAGGGGCGCGAACCCTACAG AGG 3’</td>
<td>~500</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

M. albus biological activity tests

M. albus has been previously tested for activity against a variety of plant pathogenic fungi and bacteria (Ezra et al., 2004; Ezra & Strobel, 2003; Woropong et al., 2001). The fungus’s potential activity against P. tracheiphila was tested using the procedure described by Ezra et al. (2004). M. albus was grown on one half of a two-sectioned PDA plate for 5 days prior to the addition of the test fungi to the other half of the plate. The plates were sealed with a double layer of parafilm tape and the growth of the test fungi was monitored. The growth rates of the test fungi on these plates were compared with their growth rates on control plates, in the absence of M. albus.

M. albus transformation and PCR detection

M. albus was transformed with a plasmid carrying the green florescence protein gene (gfp). Transformation was done as described by Mullins et al. (2001) and Rho et al. (2001). Transformed colonies exhibiting stable gfp activity were kept for further investigation. Specific primers were designed for PCR identification of M. albus. PCR amplification of a 500 bp product was identified with M. albus only. None of the other tested fungi reacted with the primers (Fig. 1).

Results and discussion

Isolation of endophytes

Endophytic microorganisms with inhibitory activity against P. tracheiphila and other test fungi were isolated; the majority of them, to date, are gram positive bacteria (Table 2). The search for other endophytic fungi and bacteria which may be potential biological control agents of citrus disease continues. This search for beneficial endophytes is not restricted to citrus. Some endophytes exhibiting biological activity have been isolated from other plants. Trials are currently underway to determine the optimal conditions for the reintroduction of these isolates back into fruit trees, citrus in particular. The anatomical location of the reintroduction to the plant, mode of introduction and optimal temperature and moisture conditions are all being tested. Is infiltration or injection of the microorganism sufficient for the establishing plant infection? Could irrigation with the endophytes be sufficient, or is it necessary to apply the endophytes to open wounds to ensure penetration? All of these
questions are being examined. Once we have determined the best method for reintroducing the endophyte into the plants, we will have several other challenges to address. The survival of the endophytes in the trees, their anti-pathogen activity in planta, if any, and the formulation of the most successful biological control agents will all be considered.

![Figure 1](image)


**Table 2. Biologically active isolates from trees**

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Source</th>
<th>Closest match$^\text{$}^\text{$}$</th>
<th>Level of activity$^#$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RED 1</td>
<td>Citrus roots</td>
<td><em>Serratia</em> sp.</td>
<td>High</td>
</tr>
<tr>
<td>12</td>
<td>Citrus (abandoned orchard)</td>
<td>Was not performed</td>
<td>High</td>
</tr>
<tr>
<td>15</td>
<td>Citrus (abandoned orchard)</td>
<td>Was not performed</td>
<td>Moderate</td>
</tr>
<tr>
<td>22</td>
<td>Citrus (abandoned orchard)</td>
<td><em>Bacillus subtilis</em> <em>$^</em>$</td>
<td>Moderate</td>
</tr>
<tr>
<td>38</td>
<td>Citrus (abandoned orchard)</td>
<td><em>Bacillus atrophaeus</em></td>
<td>Moderate</td>
</tr>
<tr>
<td>98</td>
<td>Citrus (abandoned orchard)</td>
<td><em>Bacillus subtilis</em> <em>$^</em>$</td>
<td>Low</td>
</tr>
<tr>
<td>B</td>
<td>Persimmon roots</td>
<td>Was not performed</td>
<td>High</td>
</tr>
<tr>
<td>C</td>
<td>Avocado</td>
<td><em>Bacillus endophyticus</em></td>
<td>Moderate</td>
</tr>
</tbody>
</table>

$^\#$ Level of activity represents the average activity measured against all the test fungi.

$^\text{\$}$ Closest match as received from the Blast software in the ICBN web site.

* Bactria morphology is different.

**Introduction of *M. albus* into citrus trees**

*P. tracheiphila* is a systemic, endophytic citrus pathogen. We chose to use this fungus as a model for other systemic fungal pathogens. *M. albus*, as an endophyte, has the potential to act as a biological control agent inside the plant. We hope that *M. albus* can serve as a systemic agent; meaning that it will spread in the plant or at least secrete its biologically active compounds to the rest of the plant via the plant transport system, controlling the pathogens’ growth and keeping the tree disease-free. As with the other microorganisms, the optimal conditions for the introduction of this fungus into the plant are yet to be determined. In order to be able to evaluate the fungus’s establishment in the plant, we had to find ways of identifying the fungus in the plant. The presence of this fungus does not induce any visible symptoms in the plant, as pathogenic fungi do (disease or HR). There were a few options: Reisolation of the fungus using the original isolate as selection agent (a method for the isolation of *M. albus* from plants was established by using the fact that it kills or inhibits all other microorganisms but its kind. (Ezra et al., 2004), PCR identification, and the use of a
visible marker. We used all of these strategies. A set of primers specific to *M. albus* was designed. These primers amplified a 500 bp fragment of *M. albus* DNA, but did not react with any other fungi tested (Fig. 1). The *gfp* gene was transferred into the fungus by *Agrobacterium*-mediated transformation and the activity of the protein was detected under bifocal microscopy. With these tools, we believe we can monitor the fungus’s establishment and progress *in planta*.

We believe that endophytes have significant potential for use as biological control agents for the control of diseases in trees, particularly fruit trees. As with other methods of biological control, this path needs to be studied, understood and adjusted for each specific purpose and case.

**References**


Evaluation of endophytic actinobacteria as antagonists of *Pythium aphanidermatum* in corn

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Abstract: Endophytic bacteria reside within plant hosts without causing disease symptoms. Suppression of plant disease due to the action of endophytic microorganisms has been demonstrated in several pathosystems. Endophytic actinobacteria isolated from healthy corn plants were assessed for their ability to control damping-off. Forty one selected isolates were screened for *in vitro* antagonism towards *Pythium aphanidermatum* and for production of endo-glucanase, pectinase and chitinase. The taxonomy characterization of the isolates was fermented by using a combination of phenotypic, genotypic and phylogenetic methods and by FAME. All isolates were assigned to the genus *Streptomyces*. Some strains were putatively identified as *S. halstedii, S. lavendulae, S. californicus, S. rochei rochei, S. anulatus, S. exfoliatus, S. glaucescens, S. albidoflavus* and *S. violaceusniger violaceusniger*. Six strains were highly related and could not be identified to any *Streptomyces* species. These strains could represent novel streptomycete species. All *Streptomyces* isolates completely inhibited the mycelial growth of the pathogen. The active compounds, extracted with ethyl acetate, produced by all isolates, also strongly inhibited the mycelial growth. A *Streptomyces lavendulae* strain16R3B, tested for its effects on biocontrol of *Pythium* in greenhouse, significantly reduced the root rot index of cucumber and corn. Some isolates produced high amount of chitinase, pectin lyase and endo-glucanase. The results of this study indicate that endophytic actinobacteria isolated from corn plants provide an advantage as biocontrol agents for use in the field, where other have failed, due to their ability to colonize internal tissues of the host plant.

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Role of arbuscular mycorrhiza-associated bacteria from the genus *Paenibacillus* in biocontrol of *Pythium*

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Abstract: Several studies have shown that arbuscular mycorrhiza (AM) can increase plant tolerance against root diseases caused by *Pythium*, which is an important pathogen in greenhouse production of cucumber. The mode of action of this biocontrol activity is not fully understood, but both direct interactions between AM fungi and *Pythium*, and mycorrhiza-mediated plant defense reactions have been proposed. In addition, antagonism from bacteria in the mycorrhizosphere has been proposed. In the present study, the *Pythium* biocontrol features of bacteria from the genus *Paenibacillus* were examined. *Paenibacillus* strains which were isolated either from the rhizosphere of mycorrhizal (symbiosis with the AM fungus *Glomus intraradices* BEG87) or non-mycorrhizal cucumber plants or from the hyphosphere of *G. intraradices* were included in the experiments.

A simple cucumber seed emergence bioassay was developed using six-wells microtiter plates with sterilized sand as the growth substrate. One cucumber seed of the variety Mystica was placed in each well, and each treatment had five replicates. Each replicate consisted of one plate with six wells. Seventeen strains of *Paenibacillus* spp. (thirteen from AM and four from non-AM systems) were screened against two *Pythium* isolates (*Py. aphanidermatum* or *Pythium* sp.), which were inoculated as one agar plug applied directly to the seed. The respective controls without *Pythium* received agar plugs without *Pythium*. After sowing the microtiter plates were incubated in a growth chamber (19-21°C and 16 h photoperiod) for seven days and 3 days later each experimental unit was scored for seed emergence.

Among the seventeen strains of *Paenibacillus* spp., no strains significantly reduced damping-off incidence caused by the rather aggressive isolate *Pythium* sp. (B5), however, thirteen strains including 4 strains of *Pa. polymyxia*, 8 strains of *Pa. macerans* and 1 strain of *Paenibacillus* sp. significantly increased the percentage of seedling emergence of seeds inoculated with *Py. aphanidermatum* (FC42). The two best strains of *Paenibacillus macerans* not only reduced pre-emergence damping-off incidence with 73%, but also gave full protection against *Py. aphanidermatum* so that 68-82% of the emerged seedlings remained healthy seven days after sowing. Interactions between these strains and AM, and their combined effects on *Py. aphanidermatum* need to be investigated to further elucidate the role of *Paenibacillus* strains in the biocontrol activity of arbuscular mycorrhiza. However, our results demonstrate a potential among bacteria from *Pa. polymyxia* and *Pa. macerans* to control pre- and post emergence damping off in cucumber caused by *Pythium*. 
Plant screening strategy to select soil and rhizosphere bacteria as biocontrol agents against white root rot of avocado

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Abstract: A plant screening strategy to detect in planta bacterial biocontrol activity has been developed. This has allowed direct selection of the most effective isolates against avocado white root rot caused by Rosellinia necatrix. Biocontrol tests were performed using avocado plants cv. Reed obtained by in vitro germinated embryos. Out of 146 isolates tested, 25 expressed a disease index less than 30% when the control disease index was at 50% (ID₅₀). Among the selected isolates, only 10 had in vitro antagonistic activity against R. necatrix. This result supports the hypothesis that potential biocontrol agents against avocado root rot are rejected when only antagonism is considered during the selection process.

Key words: Bacillus spp., Persea americana, Pseudomonas spp., Rosellinia necatrix

Introduction

Rosellinia necatrix Prill. is a soilborne pathogenic ascomycetous fungus that causes white root rot disease in a large number of plants, especially fruit trees. This pathogen is one of the most important avocado diseases in Andalusia, Spain. R. necatrix is an aggressive fungus that attacks secondary roots. It develops under the cortex by mycelial aggregations invading the entire root system. Control methods recommended in Spain include soil drench with the fungicide fluazinam or soil solarization (Pérez-Jiménez, 2006). In connection with the biological control of this pathogen, there are some fungi (Szteinberg et al., 1987; Watanabe, 1991; Mendoza-García et al., 2003) and bacteria (Yasuda & Katoh, 1989; Cazorla et al., 2006), with a strong antagonistic effect against R. necatrix. Biological control with a fungal virus, which reduced virulence of the fungus, has also been studied (Kanematsu et al., 2004). Although antagonistic to R. necatrix in vitro, when tested, biological control agents, unfortunately, showed no disease control under field conditions (Szteinberg et al., 1987). In an attempt to select more effective biocontrol agents against R. necatrix, isolates of soil and rhizosphere bacteria from andalusian avocado orchards were procured (González-Sánchez et al., 2004; Pliego et al., 2004). The results of evaluations performed to characterize their biocontrol capacity against the fungus are presented below.
Materials and methods

Bacterial and fungal isolates
Bacteria were isolated from soil and root samples collected from avocado trees located in Andalusia as previously described (González-Sánchez et al., 2004). Culturable bacterial strains were stored for preservation at –80°C in Luria-Bertani medium with 20% glycerol. For pathogen inoculations, a *R. necatrix* isolate CH53 (IFAPA’s fungal collection) was chosen. This isolate was previously characterised among different local isolates as highly virulent. Inoculum consisted of fungal colonized wheat seeds (Sztejnberg et al., 1987).

Biocontrol tests
Avocado plants cv. Reed obtained by *in vitro* germinated embryos were used for biocontrol tests. Once acclimatized, plantlets were maintained in a growth chamber under artificial conditions at average temperatures of 25ºC, artificial light (40 µE m⁻² s⁻¹) and 80% RH. The seedlings were placed in pots containing 250 ml of a perlite / vermiculite substrate (50% v/v), and maintained in a growth chamber for 8 weeks before they were used for biocontrol. Every two weeks they were fertilized with 20 ml of Bayfolan S (Bayer) at 0.2%.

For biocontrol tests, surface-disinfested avocado roots (0.06% NaOCl for 15 min) were washed and subsequently immersed for 20 min into a bacterial suspension ranging between 10⁸-10⁹ cfu/ml. Treated plants were placed into pots filled with non-sterile vermiculite and inoculated during the next 24 h with *R. necatrix*-colonized wheat seeds (2 g/l substrate) buried into the substrate at four different points from the plant collar. Bacteria were evaluated using at least five replicates. An equal number of non-bacterized, but *R. necatrix*-inoculated, plants treated similarly were left as controls. Plants were watered from the bottom. Treated plants were incubated under artificial conditions as described above. As result of root infection, aerial symptoms were observed between seven and ten days post-inoculation. Aerial symptoms were evaluated (0, healthy plant; 1 wilting of leaves; 2, over-all wilting and dryness of leaves and 3, dead plant) and a disease index percentage was recorded (Teixeira de Sousa, 1985). Bacterial strains were selected as potential biocontrol agents when they expressed a disease index less than 30% when the control disease index was at 50% (ID₅₀).

Characterization of bacterial strains
Antagonistic activity of total soil and root bacterial isolates was tested as previously described (González-Sánchez et al., 2004). Bacterial strains inhibiting *R. necatrix* mycelial growth were classified as antagonistic bacteria for further comparisons. Selected strains were identified at species level using tests API 20NE (BioMerieux, Mercy, Létoyle, France) and analysis of the 16S rDNA sequence. For this, colony PCR was performed on Gram-negative selected strains, using the primers 41F/1486r-N, and on Gram positive, using the primers 41F/1486r-P (Stackebrandt & Goodfellow, 1991). The resulting PCR fragment was purified (QIAquick PCR Purification Kit 50) and was used directly for sequencing (Macrogen Inc., Korea). The sequences were analysed and homology studies achieved using National Center for Biotechnology Information Genebank Blast software (Bethesda, MD, USA).

Results and discussion
To date, a collection with 330 bacterial strains isolated from soil and avocado roots has been obtained and tested for antagonistic activity against *R. necatrix*. From these isolates, a preliminary characterization has been carried out, and 67% were Gram negative and 33% Gram positive. Among them, 8% (26 isolates) expressed antifungal activity against *R. necatrix* (Table 1). Among antagonistic isolates 50% (13 isolates from 26) were fluorescent
on King’s B plates which indicates that they were fluorescent *Pseudomonas* spp. (data not shown).

A total of 143 isolates were selected and evaluated using a biocontrol test system on avocado plantlets. All antagonistic isolates were included. From 143 tested isolates, we selected 25 isolates (17%) because they expressed a disease index less than 30% when the control disease index was at 50% (Fig. 1). Among these 25 isolates with biocontrol potential activity, 10 had been previously characterized as *in vitro* antagonistic to *R. necatrix*.

**Table 1. Results of root and soil bacteria from avocado orchards used in this study**

<table>
<thead>
<tr>
<th>Orchard</th>
<th>Collection¹</th>
<th>Antagonistic²</th>
<th>Evaluated³</th>
<th>Selected⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram +</td>
<td>Gram -</td>
<td>Gram +</td>
<td>Gram -</td>
</tr>
<tr>
<td>EA Tropicales</td>
<td>26</td>
<td>50</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>García Martos</td>
<td>16</td>
<td>22</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>P-1 La Mayora</td>
<td>12</td>
<td>20</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Avocado Export</td>
<td>19</td>
<td>35</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Aguilar</td>
<td>8</td>
<td>15</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Olivares</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Francisco Pineda</td>
<td>9</td>
<td>13</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Tío Palomo</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Paulete</td>
<td>5</td>
<td>18</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Rafael Arcas</td>
<td>2</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>La Mayora</td>
<td>8</td>
<td>14</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Barranco</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
<td>220</td>
<td>4</td>
<td>22</td>
</tr>
</tbody>
</table>

¹ Number of bacterial isolates obtained from 50 soil and root samples from 19 avocado trees located in Málaga (Spain).
² Number of selected isolates with marked antagonistic activity against *R. necatrix*.
³ Number of isolates evaluated for biocontrol activity with avocado plants.
⁴ Number of selected isolates with biocontrol activity under experimental conditions.

**Figure 1. Disease index of selected bacterial strains with biocontrol activity against *R. necatrix***
Characterization of the selected isolates by API 20NE tests, combined with homology analyses of nucleotide sequences of PCR-amplified 16S rDNA fragments, revealed that these isolates belong to *Pseudomonas* spp. (14 strains), *Bacillus* spp. (10 strains) and *Enterobacter* sp. (1 strain). These species were: *P. chlororaphis* (CB58, CB254 and CB303), *P. putida* (CB6, CB21, CB95, CB104, CB217, CB286 and CB320), *P. fluorescens* (CB32, CB225 and CB 306), *Pseudomonas* sp. (CB78), *B. cereus* (CB171, CB176 and CB184), *B. subtilis* (CB100, CB115 and CB301), *Bacillus* spp. (CB12, CB135, CB153 and CB156) and *Enterobacter* sp. (CB240). The selected strains with antagonistic activity were: CB32, CB58, CB78, CB100, CB115, CB217, CB225, CB254, CB301 and CB303 (Fig. 1).

Using this plant screening strategy we have selected 25 strains with biocontrol potential activity from among 143 isolates tested (17%). Simultaneously, we found 10 strains with biocontrol potential activity form among 26 previously characterized antagonistic isolates (38%). Although the antagonism criteria yielded a higher percentage of strains with biocontrol potential activity than the plant screening strategy presented here, we have demonstrated that a substantial number of these strains (15 out of 25 selected strains), with no in vitro antagonistic activity, were selected by this strategy. Results support the hypothesis that potential biocontrol agents against white root rot of avocado are rejected when only antagonism is considered during the selection process. Future work will focus on elucidating mechanisms, which could be operating during plant pathogen biocontrol agent interaction. For this purpose, these selected biocontrol isolates are now being studied further in relation to production of antifungal compounds and root colonization.

References


High-Throughput preliminary screening of microorganisms with potential activity against *Plasmopara viticola* by means of quantitative Real-Time Polymerase Chain Reaction

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Abstract: Downy mildew is one of the most important grapevine diseases worldwide, caused by the obligate parasite *Plasmopara viticola*. Control of the disease is so far achieved only by fungicide applications. Biocontrol is a more natural and less environmental harmful alternative compared to chemical pesticides. Screening for biocontrol efficacy on plants is expensive and time consuming. Therefore a rapid high-throughput method was developed for relative quantification of *P. viticola* DNA directly from *Vitis vinifera* leaves by means of multiplex real-time quantitative polymerase chain reaction (PCR) with TaqMan chemistry. A total number of 254 microorganisms, (bacteria and filamentous fungi), isolated from different substrate in untreated or abandoned vineyards, were tested using the high-throughput preliminary screening of and the potential role as *Plasmopara viticola* biocontrol agents was studied. Leaf disks were cut from fresh young (less then two weeks old) grapevine leaves. The leaf disks were treated with the microorganisms and afterwards inoculated with a suspension of *P. viticola* sporangia. Untreated and copper treated references were included. After two days of incubation at 20°C the disks were freeze-dried and the DNA extracted. A real-time polymerase chain reaction was carried out and a relative quantification of *P. viticola* DNA was achieved. A high ratio between *P. viticola* DNA / *V. vinifera* DNA indicated a successful infection and a low biocontrol activity. Conversely, a low ratio indicated a good disease control. Among the 254 tested microorganisms, about 134 showed a reduction of *P. viticola* DNA compared to the untreated control, thus meaning a potential antagonistic activity. *In planta* tests should then be performed to evaluate their real activity, however this high-throughput method allows to reduce, discharging the ineffective ones, to at lest one half the number of microorganisms to be tested on plants.

Key words: biocontrol agent

Introduction

Downy mildew, caused by the obligate oomycete *Plasmopara viticola* is one of the most destructive grapevine diseases occurring worldwide. The pathogen attacks all green parts of the plant, flowers and bunches. Severe infections cause leaves and berries to shrivel and fall. Control of downy mildew infection is currently based on application of chemical treatments. In organic viticulture the most effective agent is copper but, in order to reduce its accumulation in soil, EU has limited its use in organic agriculture. Alternative disease control strategies that focus on copper replacement are currently evaluated by several researchers (e.g. European Project REPCO, www.rep-co.nl). Biocontrol is a natural and less environmentally harmful alternative compared to chemical pesticides. To the best of our knowledge no microorganism was yet isolated that could provide a protective effect against grapevine downy mildew. At the SafeCrop Centre (Istituto di San Michele all’Adige, Trento, Italy) a collection of about 1500 microorganisms (bacteria, yeasts and filamentous fungi) isolated
from untreated and wild growing grapevines and soil was established to be screened for potential biocontrol agent against \textit{P. viticola}. The aim of this study is to implement and validate a high-throughput screening method. As the bioassay on plants is expensive, time consuming and subjected to risk of cross contaminations, the putative biocontrol activity of microorganisms was assessed indirectly by estimating the \textit{P. viticola} biomass by real-time quantitative polymerase chain reaction (RT-PCR) on leaf disks (Valsesia et al., 2005).

Materials and methods

\textit{BCAs selection and leaf treatments}

Two-hundred and fifty microorganisms were tested for their potential preventive biocontrol activity against \textit{P. viticola} on artificially inoculated grapevine leaf disks. Microorganisms were isolated and maintained on potato dextrose agar (PDA, Sigma) slants at 4°C. Mycelium agar plugs were transferred to PDA Petri dishes, incubated for 15 days at 25°C and used for the preparation of the inoculum. Suspension of \textit{P. viticola} sporangia were prepared by washing sporangia from sporulating lesions on leaves with distilled water. The suspension of sporangia was adjusted to 4.8×10^5 sporangia/ml.

Leaf disks were cut from one-two week old grapevine leaves of susceptible cv. Pinot Noir. Leaf disks were dipped into a suspension of each of the 250 selected microorganisms for five minutes and then sprayed with the \textit{P. viticola} sporangia suspension. Leaf disks were incubated in 24-well plates at 20°C and 100% relative humidity for three days and freeze-dried. Four replicates (leaf disks) per microorganism (MO) were used.

\textit{DNA extraction and quantitative RealTime PCR}

DNA was extracted with NucleoSpin Multi-96 Plant Kit (Macherey-Nagel, Duren, Germany). Instead of the supplied lysis buffer, CTAB was used and instead of one final elution of 180 µl, two elution of 60 µl were made. The DNAs collected in the second elution were afterwards analysed with RT-PCR according to Valsesia et al. (2005). DNA of samples, pure \textit{P. viticola} DNA, pure Vitis vinifera DNA and no DNA template control were amplified. Multiplex reaction contained 1×TaqMan Universal Master Mix, 250 nM \textit{P. viticola} VIC-labelled probe (GiopP-VIC), 250 nM \textit{V. vinifera} FAM-labelled probe (ResP-FAM), 900 nM \textit{P. viticola} forward and reverse primers (Giop), 120 nM \textit{V. vinifera} forward and reverse primers (Res) and 5 µl template DNA. Amplification were performed with the standard short cycling parameters (50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min). Results are shown as ratio of CT \textit{V. vinifera} over CT \textit{P. viticola} (infection coefficient, IC). A high ratio (IC~1) between \textit{P. viticola} DNA / \textit{V. vinifera} DNA indicated a successful infection and tissue colonization by \textit{P. viticola} and a low control activity. Conversely, a low IC (IC~0.5) indicated a good disease control.

Table 1. Primer set used for RT-PCR quantification of \textit{Plasmora viticola} in leaf disks of \textit{Vitis vinefera}

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Primer set</th>
</tr>
</thead>
<tbody>
<tr>
<td>GiopR GGTTGCAGCTAATGGATTCTCTA</td>
<td>ResR GCTGTGCCAATGGCTAGGA</td>
</tr>
<tr>
<td>GiopF TCCTGCAATTCCATTTACGTT</td>
<td>ResF CGAGGAATTAGAAACGCTCAAC</td>
</tr>
<tr>
<td>GiopP- GCCGCCAGTTCGCAGCTTCTCA</td>
<td>ResP- TGCCAAGGGTCCGCCIAC</td>
</tr>
<tr>
<td>VIC</td>
<td>FAM</td>
</tr>
</tbody>
</table>
Results

Untreated grapevine leaf disks (infection controls, NT) were successfully inoculated with *P. viticola*. RT-PCR of NT controls showed an average IC value of 0.86±0.09 (Fig. 1). Of the 250 microorganisms tested, 69 microorganisms showed a significantly (*P*<0.05) smaller IC respect to the NT control (IC<0.79). One hundred and twenty microorganisms showed no significant difference (T test *P*>0.05) from the NT control (0.79<IC<0.93). The remaining 61 microorganisms showed a significantly higher IC. In Fig. 1 the results of 18 microorganisms are shown as an example. The lowest amount of *P. viticola* DNA was assessed after treating the four leaf disk replicates with the microorganism coded by “81F” (IC= 0.67). The non inoculated leaf disks never gave positive results for *P. viticola* DNA.

Discussion

The microorganisms tested in this assay could be classified in three main categories: potential BCAs, neutral MO and infection enhancers. This high-throughput screening allowed reducing 72.4% of them having no effect or even inducing, enhancing or favouring downy mildew infections. This quick and high-throughput method assured less time requirement and higher reliability and accuracy than the traditional bioassay screening technique (quantitative assessment of sporulation on treated leaf disks, by counting the number of sporangia/cm²), but require expensive equipment and consumables. An additional benefit of this approach
compared to the traditional leaf disk bioassay is related to short incubation time need for the analysis, which strongly reduces the risk of rots that can develop until sporulation is seen and therefore avoid the risk of false positive/negative. In the near future in planta tests will be performed to evaluate the real biocontrol activity of the promising microorganisms.

Acknowledgements

This research was supported by the project “Studi finalizzati ad ottemperare alle limitazioni dei quantitativi di rame o mediante l’impiego di formulazioni a basso dosaggio o con l’adozione di mezzi alternativi” funded by MiPAF, Italy.

Reference

Frequency, diversity and biocontrol activity of surfactant-producing *Pseudomonas* species in Vietnam

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**Abstract:** Biosurfactants are surface-active molecules produced by a variety of bacterial genera, including plant-associated *Pseudomonas* species. Among the biosurfactants produced by *Pseudomonas* species, rhamnolipids and cyclic lipopeptides (CLPs) have received considerable attention in biological control. CLPs are composed of a fatty acid tail linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain. CLPs are very diverse both structurally and in terms of their biological activity. The structural diversity is due to differences in the length and composition of the fatty acid tail, and to variations in the number, type and configuration of the amino acids in the peptide moiety. CLPs have received considerable attention for their antimicrobial, cytotoxic, and surfactant properties. For the antagonistic *Pseudomonas* species, CLPs play a key role in antimicrobial activity, motility, biofilm formation, and biological control of plant pathogenic fungi and oomycetes.

In this study, 64 samples were collected from the rhizosphere of black pepper plants grown in three different regions in the Quang Tri district in central Vietnam. Rhizosphere suspensions were plated onto semi-selective medium for fluorescent *Pseudomonas* species and a large number of randomly selected colonies were subjected to a drop collapse assay to identify putative CLP-producing *Pseudomonas* species. A total of approximately 300 isolates that produce biosurfactants were selected and subjected to genotypic analyses by BOX-PCR. A total of 65 different genotypic groups, including 42 groups with single isolates and 23 groups with multiple isolates, were identified. Subsequent chemical profiling of the biosurfactants produced by representative isolates of the 23 genotypic BOX-PCR groups resulted in the identification of only 4 different, putative CLP surfactants. The differential ability of the identified biosurfactant-producing *Pseudomonas* isolates to control phytophthora root rot of black pepper and cucumber will be presented.
Biological control of verticillium wilt of cotton by endophytic bacteria

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Abstract: Endophytic bacteria were isolated from the roots of various cotton cultivars and weeds in the cotton fields of Aydin province during 2004-2005 growing season. Twelve bacterial strains out of 158 isolates showed a significant inhibition zone towards *Verticillium dahliae* Kleb. on potato dextrose agar (PDA) plate. Twelve antagonistic strains were tested for their *in-vitro* ability of producing volatile organic compounds, siderophore, indole acetic acid and phosphate solubilization. The antagonistic strains were further evaluated for the capacity to control verticillium wilt *in vivo* and to induce growth promotion *in vitro* by bacterial seed treatment. Two fluorescent pseudomonads and one Bacillus strains significantly reduced the incidence of verticillium wilt on cotton plants.

Key words: *Verticillium dahliae*, volatile chemicals

Introduction

Endophytes are defined as those bacteria that can be isolated from surface-disinfested plant tissue or extracted from within the plant, and that do not visibly harm the plant (Hallmann et al., 1997). Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants, ranging from woody tree species to herbaceous plants (Cindy et al., 2002). Surveying of indigenous bacterial endophytes from cotton and sweet corn, McInroy & Kloeper (1995) reported that gram negative bacteria comprised 70.5% of the endophytic bacteria and 27 of the genera identified, and there were 14 taxonomic groups present in cotton roots that were not in cotton stems. Researchers have reported that endophytic bacteria have the potential to reduce fusarium wilt (Chen et al., 1995) and verticillium wilt of cotton (Hallmann et al., 1997). In Turkey, verticillium wilt is one of the most important diseases of cotton and causes great economic losses in many crops. Since the chemical control of the disease is nearly impossible, and soil solarization or broad-spectrum fumigant application is not cost effective in large cotton fields; biological control can be an environmentally friendly alternative against this devastating pathogen.

Our objectives were to isolate naturally occurring endophytic bacteria that could be applied to cotton seeds for protection verticillium wilt and to understand possible mode of actions of the bacteria as a plant growth promoter.

Material and methods

Plant and fungal material

Surface disinfested cotton seeds (*Gossypium hirsutum* cv. Sayar-314), which are sensitive to *V. dahliae* were used in all experiments. Fourteen isolates of *V. dahliae* isolated from plants with wilt symptoms in different locations of cotton growing areas of Aydin province were
tested for pathogenicity. The most pathogenic isolate was chosen in this study (data not shown). A conidial suspension of isolate VP1A of *V. dahliae* was used at a concentration of 4×10^6 conidia/ml in all *in vivo* assays.

**Isolation of endophytic bacteria**

In the 2004-2005 cotton-growing season, endophytic bacterial strains were isolated from roots of weeds and cotton plants collected from cotton fields at various locations, and the experimental plots of cotton breeding program for resistance to *V. dahliae* at the Nazilli Cotton Research Institute in Aydin province of Turkey. Plants were uprooted with rhizosphere soil and transported to the laboratory in polyethylene bags where they were thoroughly washed with tap water. The top 5-6 cm of the root segments were shaved using a handheld pencil sharpeners with two different sized holes after surface sterilization. Approximately 0.3-0.4 g of root tissue was macerated with a sterile mortar and pestle in 0.01 M phosphate buffer-PB (pH=7.2). Samples were serially diluted with PB and plated onto R2A (Oxoid). Plates were incubated for 3 days at 24°C. Depending on the sample properties, 3 to 10 colonies from each sample with different colony morphology were subcultured on *Pseudomonas* Agar F-PAF (Merck) and the obtained pure cultures were stored in the freezer at -80°C prior to assaying antagonistic activity against *in vitro* hyphal growth of *V. dahliae*.

**Inhibition of fungal growth**

Bacterial isolates were screened for their ability to produce antifungal substances against *V. dahliae* by a dual-culture *in vitro* assay on PDA. Each plate was inoculated with four droplets of 10 µl bacterial suspension (at a conc. of 10^8 cell/ml) symmetrically placed on four sites at equal distances (2 cm) from the center of plate. After 24 hours incubation at 24°C, a single 5-mm-diameter mycelial disc was placed in the center. As a control, a disc of *V. dahliae* was grown on a PDA plate. The radius of each fungal colony was measured after incubating 10 d at 24°C in darkness and the relative growth inhibition was expressed as [(treatment-control) / control×100]. The isolates causing significant inhibition were also examined for gram staining, endospore formation, and fluorescent pigment on PAF and hypersensitive reaction on White Burley tobacco leaves.

**Plant inoculation and disease evaluation**

The cotton seeds were soaked for 30 min in a bacterial suspension (100 mg bacteria and 2 ml of carboxy methyl cellulose (CMC) solution per 20 seeds) or in the same volume of CMC without bacteria (the control treatment) and then dried under a laminar flow hood (Quadt-Hallmann et al., 1997). The seed bacterization procedure resulted in mean bacterial concentrations of 1×10^8 cfu/seed as determined by dilution plating after 48 h. The bacterized and control seeds were planted in 10 cm diameter plastic pots containing an autoclaved soil-sand-peat (1:1:1) mixture and then fertilized once with liquid fertilizer (Sheffer, 16-8-24 N-P-K) 10 days after planting. Each treatment was replicated five times with two plants per replication.

Plants were inoculated at the sixth true leaf stage by using 10 µl of conidial suspension of VP1A by the method of Hanson (2000). The conidia suspension was drawn into a five-ml-syringe and with a 22-gauge-needle a single drop was applied to the plant surface on the first internode above the soil line. The needle then was used to stab through the drop into the cotton stele. Controls were inoculated with sterile water. Plants were incubated under fluorescent light (16-h light/8-h dark) at 24°C for 28 d in growth room. Each week after inoculation, the first six true leaves of the plant were individually assessed for disease severity using a 0-4 scale where 0 = no signs or symptoms of infection; 1 = 1-25% of the leaf area with symptoms (chlorosis, necrosis, or epinasty); 2 = 26-50%; 3 = 51-75%; and 4 = >75% of the leaf area with symptoms or the plant is dead. Results were combined for all leaves to determine a severity rating for the plant. The area under disease progress curve AUDPC’s
were calculated numerically using the trapezoidal rule and plotted for successive assessment dates (Campbell & Madden, 1990).

**Antagonistic mechanisms**

Siderophore production was determined using the plate CAS assay described by Schwyn & Neiland (1987). The ability of the bacterial isolates to solubilize tri-calcium orthophosphate was tested on agar plates as described by Nautiyal (1999). Quantification of IAA production of bacterial isolates was performed in the presence of tryptophan as described by Patten et al. (2002). Color development with Salkowski’s reagent was measured in a spectrophotometer at 535 nm and the concentration of IAA in each of the culture media was expressed as µg/ml by comparison with standard curve.

**Growth promotion assay**

An *in vitro* seedling test was used to determine the possible effect of endophytic bacteria and their volatile chemicals. The previously described bacterized cotton seeds (2 seeds per plate) were placed onto water agar-WA (1%) in 90 mm plastic Petri dish and incubated at 24°C to test the direct effect of the bacterium on cotton seedling germination. The growth promotion effect of volatile chemicals released from endophytic bacteria was tested in the laboratory with bipartite Petri dishes containing PDA in one side and water agar (1%) in the other. One compartment of divided Petri dish was inoculated by streaking with the bacterium and incubated at 24°C for 48 h, and the surface disinfested cotton seeds (2 seeds per plate) were transferred to the other (containing WA) side, and then covered with parafilm to minimize air and volatile chemicals exchange. The plates were incubated at 24°C in darkness. Radicle length was measured after 48 and 72 h, and the growth rate of the seedlings was determined (millimeter per day). All the experiments were performed in triplicate, and the results were expressed as mean values.

**Results and discussion**

Only twelve endophytic strains out of 158 isolated from all of the 22-weed and 23-cotton root samples collected in two-year period showed significant fungal growth suppression. Two of these endophytes (C5, E2) were confirmed to be gram-positive organisms producing endospores, and eight out of the remaining ten (E21, H9, B8, F8, B5, I3, E22, B4) were fluorescent pseudomonads. Two of the strains (F5 and F2) produced siderophore and none of the endophytes induced hypersensitive reactions on tobacco. Table 1 summarizes the results of *in vitro* and *in vivo* assays for inhibition of *V. dahliae*. Based on the performance in the *in vivo* assay, 3 bacterial isolates (E21, C5 and F5) caused significant reduction of AUDPC values of verticillium wilt.

Depending on the bacterial strains, treatment of cotton seed (cv Sayar 314) with endophytic bacteria, and volatile chemicals released from bacteria in bipartite Petri dish assay caused significant stimulation of seedling radicle growth (Table 1). Four strains (B8, B4, F5 and E21) inducing significant growth stimulation by volatile compounds had IAA-producing and phosphate solubilization (except B8) ability in laboratory conditions. Recently, Ryu et al. (2003) have found that some plant growth promoting rhizobacteria release a blend of volatile components that promote growth of *Arabidopsis thaliana*.

Our results demonstrate that seed treatment with endophytic bacteria can significantly reduce symptoms of verticillium wilt on cotton in greenhouse. The volatile compounds released from endophytic bacteria have a stimulating effect on seedling growth. Future work will test the efficacy of these biocontrol agents in controlling verticillium wilt and promoting growth under field conditions.
Table 1. *In vitro* and *in vivo* activity of bacterial endophytes on *V. dahliae*, their growth promotion effects on cotton seedling, and some of their *in vitro* metabolic characteristics

<table>
<thead>
<tr>
<th>Strain No</th>
<th>Origin of plant species and (cv)</th>
<th>% Hyphal growth inhibition</th>
<th>Relative AUDPC&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Radicle growth rate of cotton seeds (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>IAA µg/ml</th>
<th>P Soluble (mm)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>E21</td>
<td><em>G. hirsutum</em> (DPL882)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td><em>G. barbadense</em> (Giza 45)</td>
<td>151.4 b</td>
<td>45.7 e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>92.5 de&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.4</td>
<td>10</td>
</tr>
<tr>
<td>F5</td>
<td><em>G. hirsutum</em> (Stonville 825)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9</td>
<td><em>G. barbadense</em> (Askabat 100)</td>
<td>163.0 a</td>
<td>74.3 cde</td>
<td>108.2 bc</td>
<td>0.1</td>
<td>7</td>
</tr>
<tr>
<td>B8</td>
<td><em>G. hirsutum</em> (Tamcot CD3H)</td>
<td>127.3 d</td>
<td>97.1 bcd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F8</td>
<td><em>G. hirsutum</em> (Stonville 825)</td>
<td>145.4 bc</td>
<td>101.4 bcd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B5</td>
<td><em>G. hirsutum</em> (Tamcot CD3H)</td>
<td>138.9 c</td>
<td>115.7 abc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I3</td>
<td><em>G. hirsutum</em> (Gedera 5)</td>
<td>127.8 d</td>
<td>118.6 ab</td>
<td>94.3 de</td>
<td>1.6</td>
<td>9</td>
</tr>
<tr>
<td>E22</td>
<td><em>G. hirsutum</em> (DPL882)</td>
<td>139.2 c</td>
<td>124.3 ab</td>
<td>99.9 cd</td>
<td>1.6</td>
<td>9</td>
</tr>
<tr>
<td>B4</td>
<td><em>G. hirsutum</em> (Tamcot CD3H)</td>
<td>140.6 c</td>
<td>135.7 ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td><em>G. hirsutum</em> (GC 8915)</td>
<td>128.9 d</td>
<td>145.7 a</td>
<td>110.3 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td><em>G. hirsutum</em> (Stonville 825)</td>
<td>146.5 a</td>
<td>151.4 a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con.</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<sup>1</sup> Relative AUDPC was calculated for each treatment as the percent of the AUDPC (as 100%) of the non-treated control.

<sup>2</sup> Diameter of the clear zone around the bacterial colony (mean of the three replicates).

<sup>3</sup> Means within each column followed by the same letter are not significantly different according to Fisher’s protected least significant difference test at *P*=0.05.

References


Characterization of new strains of native bacteria that protect grapevine leaves against Botrytis cinerea and induce plant defense reactions

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Abstract: 282 bacteria were isolated from the rhizosphere and organs of healthy field grown-grapevine plants and screened for their ability to protect grapevine leaves against Botrytis cinerea. A set of 26 strains was shown to confer a strong protection against the pathogen. Phenotypic and molecular analysis identified seven of these strains as subgroups of Pseudomonas fluorescens (PTA-268, PTA-CT2), Bacillus subtilis (PTA-271), Pantoea agglomerans (PTA-AF1, PTA-AF2) and Acinetobacter lwoffii (PTA-113, PTA-152). The biocontrol activity of these strains was correlated with differential induction of lipoxygenase, phenylalanine ammonia-lyase and chitinase activities in grapevine leaves, as defense markers. In vitro antifungal experiments further indicated that only PTA-AF1 and PTA-CT2 exerted also a direct antagonism against B. cinerea.

Key words: biocontrol, gray mold, induced resistance, rhizobacteria

Introduction

Grapevine (Vitis vinifera L.) is highly vulnerable to gray mold caused by Botrytis cinerea, which is currently controlled by fungicides. Related environmental problems and pathogens developing resistance have promoted the consideration of biological control and induction of plant resistance strategies by using plant-associated microorganisms.

Most of plant-associated bacteria are biocontrol agents (BCA) against fungal pathogens. The biocontrol activity depends on a wide variety of traits, as the production by BCA of antifungal compounds and induction of resistance in the host plant. Bacteria-induced systemic resistance (ISR) has been demonstrated in a variety of plant species (Van Loon et al., 1998), and is effective against a broad spectrum of pathogens. In some cases, ISR is associated with an accumulation of pathogenesis-related (PR) proteins (Maurohofer et al., 1994), some of which have antifungal activity and therefore contribute to plant resistance (van Loon & van Strien, 1999). In other cases ISR has been reported to be independent of PR protein expression (van Wees et al., 1997; Pieterse et al., 1998). The relative importance of all these mechanisms depends strongly on strains of BCA and plant genotype.

The characterization and use of grapevine-associated bacteria as BCA against gray mold has not been reported yet. Here, we screened non-pathogenic grapevine-associated bacteria for biocontrol ability against a highly virulent B. cinerea by using detached leaves from in vitro-grown plantlets. Phenotypic and molecular analyses were performed to identify seven bacterial strains exhibiting high protection of grapevine leaves against B. cinerea. Some mechanisms by which selected bacteria may protect grapevine against B. cinerea (antagonism against the pathogen and induction of plant defense reactions) were also investigated.
Materials and methods

Isolation of bacterial strains and protection assays
Bacterial strains were isolated from the rhizosphere, roots, leaves and stems of field-grown grapevine (*Vitis vinifera* L., cv Chardonnay) as described by Trotel-Aziz et al. (2006). Isolated bacteria were cultured on Luria Bertani agar and screened for their protective effect against a highly virulent *Botrytis cinerea* (strain 630) using detached leaves from in vitro-grown grapevine plantlets (Trotel-Aziz et al., 2006).

Identification of the bacteria exhibiting high biocontrol activity
Identification of bacteria was first performed with the standardized micro-methods API 20E, 20NE and 50CHB as described by the manufacturer (BioMérieux). According to their origin, phenotypic characteristics, and biocontrol efficacy, seven strains were further identified by sequencing *rrs* gene of 16S rRNA at the Pasteur Institute (CIMB, Paris, France) as described by Trotel-Aziz et al. (2006). Phylogenetic trees were generated by Neighbor-joining program.

Determination of plant defense reactions
The relevance of the newly identified bacteria as inducers of defense responses in grapevine leaves was examined by monitoring the activity of lipoxygenase (LOX), phenylalanine ammonia lyase (PAL), and chitinase, as markers of plant defense. Detached leaves from in vitro grown plantlets were incubated in the presence of the selected bacteria and enzyme activities were determined at different times. LOX activity was assayed by measuring absorption arising from the production of fatty acid hydroperoxydes (Axelrod et al., 1981), PAL activity was determined by measuring the formation of cinnamic acid according to the method of Tanaka et al. (1974), and chitinase activity was determined as described by Wirth & Wolf (1992) using carboxymethyl/chitin/remazol brillant violet 5R as a substrate.

Antagonistic activity
Petri plates containing PDA medium were inoculated in the center with bacterial suspension (10^8 CFU/ml), and *B. cinerea* (strain 630) was placed surrounding bacterial inoculum. After 7 days at 22°C bacteria that secrete an antifungal substance may prevent fungal mycelium growth, resulting in an inhibition zone around the bacterial colony.

Results and discussion

Screening of grapevine-associated bacteria for biocontrol activity against *B. cinerea*
Based on abundance and phenotypic criteria, a total of 282 isolated bacteria were screened for their protective effect against a highly virulent *B. cinerea* using detached leaves from in vitro-grown grapevine plantlets. Overall, 26 isolates strongly reduced disease development on leaves compared to the non-bacterized control, which showed severe disease symptoms. In most cases, the reduction of necrotic lesion varied from 61 to 87%.

Identification of some bacteria exhibiting high biocontrol activity
Extensive phenotypic characterization with protective strains and results obtained with the standarized API test systems made it possible to identify most isolates as putative *Acinetobacter* sp., *Bacillus* sp., *Pantoea* sp. and *Pseudomonas* sp. strains (Table 1). Furthermore, the 16S rRNA gene sequences (EMBL database) of seven selected strains (Table 1) identified them as distinct *Acinetobacter lwoffii* (PTA-113 and PTA-152: AM293675 and AM293676), *B. subtilis* (PTA-271: AM293677), *P. fluorescens* HhSoUsc group (PTA-268: AM293678), *P. fluorescens* S2 group (PTA-CT2: AM293679), and *Pantoea agglomerans* (PTA-AF1 and PTA-AF2: AM293680 and AM293681). Related phylogenetic trees indicated that identified bacteria are new biocontrol agents against *B. cinerea*. 
Table 1. Phenotypic and molecular identification, and antagonistic activity of seven selected bacteria isolated from the rhizosphere and organs of healthy grapevine plants with biocontrol ability against *Botrytis cinerea*

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Source</th>
<th>Gram reaction</th>
<th>API 20E/20NE/50CHB</th>
<th>16S RNA sequencing</th>
<th>Antagonistic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTA-113</td>
<td>Roots</td>
<td>–</td>
<td><em>Acinetobacter sp</em></td>
<td>Acinetobacter <em>lwoffii</em></td>
<td>–</td>
</tr>
<tr>
<td>PTA-152</td>
<td>Roots</td>
<td>–</td>
<td><em>Acinetobacter sp</em></td>
<td>Acinetobacter <em>lwoffii</em></td>
<td>–</td>
</tr>
<tr>
<td>PTA-268</td>
<td>Soil</td>
<td>–</td>
<td><em>Pseudomonas fluorescens</em></td>
<td><em>Pseudomonas fluorescens</em></td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HhSoUsC group</td>
<td></td>
</tr>
<tr>
<td>PTA-271</td>
<td>Soil</td>
<td>+</td>
<td><em>Bacillus subtilis</em></td>
<td><em>Bacillus subtilis</em></td>
<td>–</td>
</tr>
<tr>
<td>PTA-AF1</td>
<td>Leaves</td>
<td>–</td>
<td><em>Pantoea sp</em></td>
<td><em>Pantoea agglomerans</em></td>
<td>+</td>
</tr>
<tr>
<td>PTA-AF2</td>
<td>Leaves</td>
<td>–</td>
<td><em>Pantoea sp</em></td>
<td><em>Pantoea agglomerans</em></td>
<td>–</td>
</tr>
<tr>
<td>PTA-CT2</td>
<td>Stems</td>
<td>–</td>
<td><em>Pseudomonas fluorescens</em></td>
<td><em>Pseudomonas fluorescens</em></td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>S2 group</td>
<td></td>
</tr>
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</table>

**Mechanisms of biocontrol by selected bacteria**

The reduction of gray mold symptoms on leaves by selected bacteria was clearly associated with early induction of grapevine defense reactions (Fig. 1). LOX, PAL and chitinase activities were stimulated in all bacterized leaves, with a range depending on the bacterial genus (Fig. 1). The maximal induction of LOX was reached after 6 to 8 h of incubation. Maximum LOX activity was achieved with the *Acinetobacter lwoffii* strains. PAL activity appeared in most cases biphasic. The first peak (after 6 to 8 h) was slightly higher in leaves treated with *P. agglomerans* PTA-AF1 and *B. subtilis*. The second peak (after 16 h) seemed highest with *B. subtilis*. Chitinase activity was stimulated after 8 h. Thereafter, chitinase activity decreased progressively. The induction of chitinase by both *P. agglomerans* strains and *P. fluorescens* PTA-CT2 was not as important. Using an antifungal activity plate assay, some of these selected bacteria (PTA-AF1 and PTA-CT2) were further shown to inhibit *B. cinerea* growth in dual culture, while the other strains did not show any antagonistic activity (Table 1).

Our results highlight the dual properties of some of these selected bacteria, which are capable of exerting direct antifungal effect and inducing plant resistance, while the others were found to induce only plant defense reactions at least partly via distinct signaling pathways. Such differential responses might be dependent on the nature of the signal compounds produced by each bacterial strain during interaction with the plant. This study opens the way to new detailed investigations concerning induced resistance in grapevine by native bacteria. Determinants of induced plant resistance are currently under investigation.

**Acknowledgments**

This work was supported by a grant from Europol’Agro (Reims, France).
Figure 1. Time courses of LOX, PAL and chitinase activities in grapevine leaves treated with bacterial strains of Acinetobacter lwoffii (A) PTA-113 (solid triangle) and PTA-152 (open triangle), Pseudomonas fluorescens (B) PTA-268 (solid circle) and PTA-CT2 (open circle), Pantoea agglomerans (C) PTA-AF1 (solid square) and PTA-AF2 (open square) and Bacillus subtilis (D) PTA-271 (solid diamond), or buffer control (dashed line). Each bacterial strain was applied at 10^7 CFU/ml at 0 time. Data are means of three replicates for each treatment. Bars represent standard deviations.

References


Swiss wheat varieties differentially attract naturally occurring *Pseudomonas* spp. in a soil dependent manner

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**Abstract:** Improvement of plant fitness and yield by natural occurring root colonizing microorganisms is of special value in low-input or organic wheat production. Beneficial soil bacteria such as certain *Pseudomonas* strains are known to promote plant growth by several mechanisms. Thus, these microorganisms are able to circumvent potential negative consequences of low-input cropping systems such as the limited supply of nutrients and higher disease pressures often observed. A significant potential exists to further improve beneficial effects by breeding wheat genotypes with a greater capacity to sustain the interactions with these bacteria. However, the interaction of crop plants (e.g. at the variety level) and bacteria as well as the conditions which favor the accumulation of beneficial microorganisms are largely unknown. Therefore, a main goal of this study was to obtain essential information about the impact of wheat genotypes on the frequency and genetic diversity of beneficial *Pseudomonas* spp. in low input soils. Three Swiss wheat varieties with a different genetic background were examined for the traits mentioned above in two distinct soils and bacteria were isolated from three different ecological niches, soil, root surface and the inside of the roots. We could show that the wheat varieties differed in the accumulation of pseudomonads carrying the *phlD* gene (which is essential for the production of the antimicrobial compound 2,4-diacetylphloroglucinol and therefore disease suppression). Furthermore, a significant interaction between soil origin and wheat variety on root colonization by *Pseudomonas* spp. was found.
Endophytic bacteria for biocontrol of coffee leaf rust (*Hemileia vastatrix*)

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**Abstract:** Suppression of plant diseases due to the action of endophytic microorganisms has been demonstrated in several pathosystems. Several mechanisms may control the suppression of plant pathogens, either directly by antibiosis and competition, or indirectly by induction of plant resistance response. The objective of this work was to select endophytic bacterial strains from coffee leaves (F), roots (R) and stems (G) with biocontrol potential against coffee leaf rust (*Hemileia vastatrix*). Two hundred fifteen endophytic bacterial strains were evaluated in coffee leaf discs. Bacterial suspensions were applied on leaf discs, 72 and 24 hours before, after and simultaneously with the pathogen. Nine bacterial strains (116G, 123G, 36F, 137G, 14F, 109G, 115G, 3F, and 119G) showed to be effective in reducing the rust development. These selected bacterial strains were evaluated in coffee seedlings (*Coffea arabica* ‘Mundo Novo’). The bacterial suspensions were sprayed to the foliage 72 and 24 hours before, after, and simultaneously with the pathogen. The best control levels were obtained when the biocontrol agents were applied 72 hours before the pathogen. Four endophytic strains - 119G, 3F, 115G, and 109G - were effective in controlling coffee leaf rust (89, 84, 69 and 66%, respectively). The activity of enzymes (peroxidase, lipoxygenase, and phenylalanine ammonia-lyase) was assessed in relation to the control of *H. vastatrix* in leaf coffee seedlings seven days after the spray of four bacterial strains (119G, 3F, 115G, and 109G). It was observed that the inoculation of the strains 3F and 119G increased the peroxidase activity in leaves of coffee seedlings and significantly reduced the number of rust lesions per leaf. The other enzymes were not affected. The detection of peroxidase activity in leaves without the presence of the antagonists and pathogen proves the induction of systemic resistance, but probably there are other mechanisms of action. The isolates were identified based on cell membrane fatty acid contents, analyzed in a gas chromatograph, using microbial identification software (MIDI Sherlock TSBA Library version 5.0, Microbial ID, USA), as 3F=*Brevibacillus choschinensis*, 115G=*Microbacterium testaceum*, and 119G=*Cedecea davisae*.

**Key words:** biological control, *Coffea* spp.

**Introduction**

Beneficial endophytic microorganisms comprise especially fungi and bacteria that colonize internal plant tissues without causing visible damage to their hosts (Petrini, 1991). They are different from phytopathogenic microorganisms because they are not detrimental, do not cause diseases to plants, and are distinct from epiphytic microorganisms, which live on the surface of plant organs and tissues (Halmann et al., 1997). Endophytic bacteria are able to penetrate and become systemically disseminated in the host plant, actively colonizing the apoplast (Quadt-Hallmann et al., 1997b), conducting vessels (Hallmann et al., 1997), and occasionally the intracellular spaces (Quadt-Hallmann et al., 1997a). This colonization presents an ecological niche, similar to that occupied by plant pathogens, and these endophytic bacteria can therefore, act as biocontrol agents against pathogens (Hallmann et al.,
The suppression of plant diseases due to the action of endophytic bacteria has been demonstrated in several patho-systems. Several mechanisms may control the suppression of plant pathogens, either directly by antibiosis and competition, or indirectly by induction of plant resistance response.

The coffee leaf rust caused by *Hemileia vastatrix* is the main disease in coffee, causing yield losses of 35 to 40%, on average. Control is basically achieved by fungicides. In 2000, in Brazil, the use of fungicides in coffee stood for 3,680 t of active ingredient (Campanhola & Bettiol, 2003). Therefore, alternatives control of coffee leaf rust must be sought. The objective of this work was to select endophytic bacteria strains from coffee leaves (F) and stems (G) with biocontrol potential against coffee leaf rust.

**Material and methods**

Bacterial isolates from leaves, and stems (Nunes, 2004), of *Coffea arabica* and *Coffea robusta* plants from São Paulo State, Brazil, were maintained in the culture collection of the Laboratory of Environmental Microbiology, Embrapa Environment. Two hundred fifteen isolates were evaluated in relation to their potential against coffee leaf rust.

Discs of young and completely developed leaves of *C. arabica* cv. Mundo Novo plants (susceptible to all *H. vastatrix* strains) were removed with a 2 cm diameter cork punch and placed into plastic boxes, abaxial surface facing up, over a layer of foam saturated with water. Bacterial suspensions were applied on leaf discs, 72 and 24 hours before, after and simultaneously with the same volume (25 µl) of *H. vastatrix* urediniospores suspension (1 mg ml⁻¹). After inoculation, boxes were covered with glass plates and incubated in the dark for 24 hours. Then, the boxes were maintained under 12-h photoperiod, 500-1000 lux, 22±2°C, and approximately 100% relative humidity. The experiment was set up in an completely randomized design (n=3), represented by nine leaf discs each. Severity of the disease was evaluated 30 days after inoculation, using a rating scale from 1 to 5, according to the percentage of leaf area with lesions (0 = 0%; 1 = 0-2.5%; 2 = 2.5-5%; 3 = 5-15%; 4 = 15-25%; and 5 ≥ 25% of leaf area with lesions).

Nine strains, 116G, 123G, 36F, 137G, 14F, 109G, 115G, 3F, and 119G, that showed to be effective in reducing the rust were then evaluated in coffee seedlings (*C. arabica* cv. Mundo Novo). The bacterial suspensions [*A550=0.1 (10⁸ CFU ml⁻¹)] were sprayed to the foliage 72 and 24 hours before, after, and simultaneously with the pathogen. After inoculation with the *H. vastatrix* urediniospores suspension (1 mg ml⁻¹), plants were incubated in the dark for 48 hours at 22±2°C at 100% relative humidity and then transferred to a greenhouse. Plants were irrigated daily and after 30 days the number of lesions per inoculated leaf was evaluated. Sterilized water was used as control. Trial was set up in a randomized blocks (n=10).

The activity of enzymes (peroxidase, lipoxygenase, and phenylalanine ammonia-lyase) was assessed in relation to the control of *H. vastatrix* in leaf coffee seedlings seven days after the spray of four bacterial strains (119G, 3F, 115G, and 109G).

The isolates were identified based on the cell membrane fatty acid contents (FAME), analyzed in a gas chromatograph, using microbial identification software (MIDI Sherlock TSBA Library version 5.0, Microbial ID, USA).

**Results and discussion**

In the test with coffee plants, the endophytic bacterial strains were not effective in controlling coffee leaf rust when applied after inoculation of the pathogen. The best control levels were obtained when the biocontrol agents were applied 72 hours before the pathogen. Four endophytic strains - 119G, 3F, 115G, and 109G - were effective in controlling the coffee leaf rust (89, 84, 69, and 66%, respectively). It was observed that the inoculation of the strains 3F and 119G increased the peroxidase activity in leaves of coffee seedlings and significantly reduced the number of rust lesions per leaf. The other enzymes were not affected (Table 1). The isolates were identified as 3F= *Brevibacillus choschinensis*, 115G= *Microbacterium testaceum*, and 119G= *Cedecea davisae*, based on cell membrane fatty acid contents.

The efficiency of certain endophytic bacterial strain in controlling coffee leaf rust can vary according to the time of biocontrol agent application. In general, the endophytes were more effective when applied 72 before inoculation of *H. vastatrix* urediniospores. The fact that the endophytic isolates showed activity when applied before the pathogen suggests that these isolates may act by antibiosis, lysis of pathogen structures, competition, or induction of systemic resistance in the host. The detection of peroxidase activity in leaves without the presence of the antagonists and pathogen proved the induction of systemic resistance, but probably there are other mechanisms of action involved (Table 1).

Even though a relatively small number of endophytic bacteria were tested, promising results were obtained regarding the selection of coffee leaf rust biocontrol agents. Further field studies must be conducted in order to analyze the real potential of endophytic bacteria in field conditions. Studies are also needed to determine the action mechanisms of those bacteria, the population density of the applied endophytes, and the best form of introduction into the host.

Table 1. Effect of endophytic bacteria on lypoxygenase, phenylalanine ammonia-lyase, and peroxidase activity in coffee seedlings (*Coffea arabica* cv. Mundo Novo) and on disease

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lypoxygenase(^1)</th>
<th>Phenylalanine ammonia-lyase(^2)</th>
<th>Peroxidase(^3)</th>
<th>Disease control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3F</td>
<td>0.18 a</td>
<td>38 a</td>
<td>96 a</td>
<td>84</td>
</tr>
<tr>
<td>109G</td>
<td>0.15 a</td>
<td>29 a</td>
<td>89 a</td>
<td>66</td>
</tr>
<tr>
<td>115G</td>
<td>0.14 a</td>
<td>36 a</td>
<td>58.b</td>
<td>69</td>
</tr>
<tr>
<td>119G</td>
<td>0.13 a</td>
<td>35 a</td>
<td>54 b</td>
<td>89</td>
</tr>
<tr>
<td>Control</td>
<td>0.13 a</td>
<td>32 a</td>
<td>50 b</td>
<td>-</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not significantly different (Tukey 5%). The percentage of disease control of the disease is relative to the control treatment obtained when the biocontrol agents were applied 72 hours before the pathogen.

\(^1\)µmol min\(^{-1}\) protein mg\(^{-1}\).

\(^2\)∆ absorbance unit min\(^{-1}\) protein mg\(^{-1}\).

\(^3\)nmol cinamic acid min\(^{-1}\) protein mg\(^{-1}\).

**Acknowledgements**

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References


Integrated approaches
Prospects for integrated management of *Sclerotinia sclerotiorum* in lettuce

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Abstract: Three biopesticide prototypes based on previously selected isolates of *Clonostachys rosea* (Cc001), *Trichoderma koningii* (Th003) and *Pichia onychis* (Lv031) were evaluated in a farm with history of losses caused by *Sclerotinia sclerotiorum*. The biological control agents achieved significant control of the disease, expressed as reduction in the number of dead plants compared with the untreated control which presented 32% of dead plants. Both IPM and Th003 treatments reduced the disease by 91 and 92% respectively, while chemical, Cc001 and Lv027 treatments reduced the disease by 28, 32 and 23%, respectively. Considering that IPM and Th003 treatments presented significant differences in reduction of disease as compared with the chemical control, they were selected to be validated in the same experimental area with a new lettuce crop. In this new experiment white mold was considerably high in the untreated control (85% of dead plants). Nevertheless, treatments significantly reduced the disease as compared with untreated control. The IPM and Th003 treatments resulted in 86 and 82% disease reduction, respectively as compared with chemical treatment that resulted in 49% reduction.

Keywords: *Clonostachys rosea*, *Lactuca sativa*, *Pichia onychis*, *Trichoderma koningii*

Introduction

*Sclerotinia sclerotiorum* is a major soilborne plant pathogen on many plant species causing economic damage (Agrios, 2005). It represents a serious threat to lettuce production in Colombia. Measures of control of this disease such as the use of chemical fungicides do not always result in consistent disease control (Hubbard & Subbarao, 1997). In previous field trials, a biopesticide prototypes based on *C. rosea* (Cc001) and *T. koningii* (Th003) showed high effectiveness in the control of *S. sclerotiorum* in lettuce under field conditions (Villamizar et al., 2004) and the yeast *P. onychis* (Lv031) was highly effective in the control of *Sclerotium cepivorum* in onion under greenhouse conditions (García et al., 2001). In the present work the effect of these biocontrol agents (BCAs), was compared with a chemical and an IPM treatment including the *T. koningii* biopesticide prototype, for controlling *Sclerotinia* disease in two sequential field lettuce crops, was investigated.

Materials and methods

A two years (2004 and 2005) field study was conducted to determine the effectiveness of biopesticide prototypes based on the antagonistic fungi *T. koningii* Th003, *C. rosea* Cc001
and *P. onychis* Lv031. Lettuce field trials were set up in a *S. sclerotiorum* naturally infested soil at the National University of Colombia (Marengo Experimental Center), with two experiments.

The first trial consisted of six treatments with each BCA applied every 8 days and independently evaluated, an IPM treatment consisting of *T. koningii* Th003 applications every 15 days combined with soil solarization and removal of dead plants, a chemical control by using the fungicides currently applied by farmers (Benomyl 200 g.Ha\(^{-1}\) and Mancozeb 1 kg.Ha\(^{-1}\)) and an untreated control. Plots were planted with four weeks old transplants (*Lactuca sativa* c.v. Coolguard) obtained from seeds primed in the presence of every BCA. The biopesticide prototypes were applied weekly as pre-planting treatments until one week before harvest. Granular formulations based upon *T. koningii* Th003 and *C. rosea* Cc001 at a concentration of 1×10\(^6\) conidia.mL\(^{-1}\) were weekly used for drench applications during one month after transplanting. Afterwards, wettable powders based on these fungi were used for foliar applications. The yeast was used as granular formulation at a concentration of 1×10\(^7\) cells.mL\(^{-1}\). In the IPM treatment, Th003 was applied every two weeks at the mentioned concentration. In the second trial the treatments comprised of a) untreated control; b) standard *T. koningii* only; c) the IPM treatment which included *T. koningii* and d) a chemical control as mentioned above. The experiments were arranged in a randomized block design, with 136 plants per plot in the first experiment and 200 plants per plot in the second one (1.8×10\(^m\) and 4.1×10\(^m\) plot size respectively) and six replicate plots. Plants were fertilized and irrigated as needed.

Figure 1. Effect of different biocontrol treatments on the percentage of *Sclerotinia* diseased lettuce plants in two consecutive years (left 2004 and right 2005)
Plants mortality and disease incidence was assessed since the first appearance of disease at 8 and 3 days intervals in the first and second experiments respectively until harvest and with the estimated yield. The treatments were compared statistically by means of Fisher’s protected least significant difference test at \( P<0.05 \).

**Results and discussion**

Percentage of disease incidence in the untreated control was lower in the first field trial than that of the second one, with 77 and 93% respectively. In the first experiment white mold disease was observed with similar intensity between 6.6 to 9.4% in all the treatments until 56 days after planting (Fig. 1), since then considerable differences were observed in disease control between treatments. Disease caused by *S. sclerotiorum* and plant mortality were significantly reduced by all treatments as compared with untreated control. However chemical control, Cc001 and Lv031 treatments were significantly less effective than treatments where *T. koningii* was applied as IPM or as individual treatment. These treatments significantly reduced plant mortality caused by *S. sclerotiorum* by 91 and 92%, respectively and diseased plants by 81 and 80%, respectively, as compared with the untreated control. Yield was significantly higher in the IPM (39 Ton.Ha\(^{-1}\)) and Th003 (33 Ton.Ha\(^{-1}\)) treatments, while in untreated control, chemical control, *P. onychis* and *C. rosea* yield was 6, 15, 15 and 16 Ton.Ha\(^{-1}\), respectively (Table 1).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Untreated control</th>
<th>Lv031</th>
<th>Cc001</th>
<th>Chemical control</th>
<th>Th003</th>
<th>IPM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incidence (%)</strong></td>
<td>76,7a</td>
<td>52,4b</td>
<td>44,4b</td>
<td>52,1b</td>
<td>15,3c</td>
<td>14,5c</td>
</tr>
<tr>
<td><strong>Plant mortality (%)</strong></td>
<td>31,6a</td>
<td>24,5b</td>
<td>21,4b</td>
<td>22,7b</td>
<td>2,6c</td>
<td>2,9c</td>
</tr>
<tr>
<td><strong>Efficacy control (%)</strong></td>
<td>-</td>
<td>23</td>
<td>32</td>
<td>28</td>
<td>92</td>
<td>91</td>
</tr>
<tr>
<td><strong>Yield (Ton.Ha(^{-1}))</strong></td>
<td>6,2c</td>
<td>15,2b</td>
<td>15,6b</td>
<td>14,7b</td>
<td>33,5a</td>
<td>38,7a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Untreated control</th>
<th>Lv031</th>
<th>Cc001</th>
<th>Chemical control</th>
<th>Th003</th>
<th>IPM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incidence (%)</strong></td>
<td>93,2a</td>
<td>-</td>
<td>-</td>
<td>80,4a</td>
<td>17,5b</td>
<td>13,2b</td>
</tr>
<tr>
<td><strong>Plant mortality (%)</strong></td>
<td>85,4a</td>
<td>-</td>
<td>-</td>
<td>58,4b</td>
<td>10,4c</td>
<td>8,4c</td>
</tr>
<tr>
<td><strong>Efficacy control (%)</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>31,6</td>
<td>87,8</td>
<td>90,2</td>
</tr>
<tr>
<td><strong>Yield (Ton.Ha(^{-1}))</strong></td>
<td>0,86c</td>
<td>-</td>
<td>-</td>
<td>7,9b</td>
<td>33,5a</td>
<td>40,5a</td>
</tr>
</tbody>
</table>

\( ^x \) Data correspond to those recorded at 71 and 76 days from transplanting in first and second experiments respectively

\( ^z \) In respect to plant mortality at the end of the experiment Values within the same row followed by different letters are significantly different \((P \leq 0.05)\) as determined by means of Fisher’s protected least significant difference test (LSD).

Due to the promising results of the IPM and Th003 treatments, they were selected to be evaluated in the second trial, including the chemical control and untreated control. In this experiment the disease was observed thirty six days after planting and was developed more
rapidly and with more intensity than in the previous year because of high rainfall (Fig. 1). The IPM and Th003 treatments significantly reduced the disease as compared with the chemical control and the untreated control (Table 1). IPM and Th003 reduced plant mortality in 90 and 88% and diseased plants in 86 and 81% respectively at the end of experiment. Yield obtained in these treatments was 41 and 34 Ton.Ha\(^{-1}\) respectively. Cultural practices as soil solarization and removal of death plants with \textit{T. koningii} applications every 15 days in the IPM treatment were used to reduce the pathogen inoculum and to prevent the spread of the pathogen from one host to another by killing the pathogen or by providing unfavorable conditions for its development.

The practices were effective in controlling \textit{S. sclerotiorum} since a long term health and viability of the plants was obtained. The BCA \textit{T. koningii} used in the present work applied every 8 days also provided effective control of \textit{S. sclerotiorum}. This microorganism probably inhibited the growth of \textit{S. sclerotiorum} by destroying existing sclerotia while inhibiting the formation of new sclerotia (Robertii et al., 2001). To validate these results trials in different commercial lettuce production plots are currently being conducted.

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**References**


Integrated approach to enhance biocontrol efficacy of post-harvest biocontrol agents

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Abstract: The past decade has seen a steady increase in seeking alternatives to synthetic fungicides for post-harvest disease control. In particular, this has led to considerable research on the use of microbial antagonists as protective agents in much the same way as packing houses use synthetic fungicides for disease control. Several biological products, based on either yeast or bacteria have been developed and commercially tested. The success and wide spread use of these products, however, remains limited. This is for several reasons, among which is the inconsistency, variability of the efficacy under commercial conditions, and the lack of understanding how to adapt “biological approaches” to crop systems in a commercial setting.

The yeast antagonist Metschnikowia fructicola was jointly developed by ARO and AgroGreen Inc. and commercialized under the trade name "Shemer". Its efficacy against pre- and post-harvest diseases was commercially tested on wide variety of fruits and vegetables. In most situations performance of the product, as a stand alone treatment, was comparable with chemical control. In others, enhancement of efficacy of the biocontrol product was required and achieved by integrating the biocontrol product with other approaches.

In recent years we have been developing an integrative approach in which various physical and biological treatments are implied. These were tailored to the specific crop, and consisted of treatments that can be integrated in the routine post-harvest practices of the crop. This approach relies on first application of hot water treatment that disinfects and eradicates existing infections followed by microbial antagonists that protect the commodity from future infections. Combining antagonists with food-grade preservatives as well as modified atmosphere packaging (MA) has been also evaluated. Tests of this integrated approach were carried out on sweet potatoes, peaches, strawberries, and citrus. Collectively the results clearly show that performance of yeast biocontrol agents could be markedly augmented by the combination of complementary approaches such as physical disinfection and additives.
Pre-harvest application of a combined treatment of *Candida sake* (CPA-1) and *Pseudomonas syringae* (CPA-5) to control post-harvest decay of pome fruits

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Abstract: ‘Golden Delicious’ apples and ‘Blanquilla’ pears were wounded in the field 2 days before harvest and treated with *C. sake* CPA-1 and *P. syringae* CPA-5 alone or in combination. After harvest fruits were inoculated with *P. expansum* and stored at 1°C for 4 months. All treatments reduced decay caused by the pathogen, but the combination of the antagonist tends to be more effective.

Key-words: antagonist mixture, apples, enhanced biocontrol activity, pears, *Penicillium expansum*

Introduction

The development of resistance of fungal pathogens to fungicides and the growing public concern toward human health and environmental respect have resulted in a significant increase in the research of alternative non-chemical treatments to control diseases. The development of biocontrol agents has been the most study area in the last few years, as indicated by a number of commercial products on the market. The yeast *Candida sake* CPA-1 (Viñas et al., 1998) and the bacterium *Pseudomonas syringae* CPA-5 (Nunes et al., 2005), were isolated from apple surface. These microorganisms have been tested, for many years, for their control activity against the major postharvest diseases of pome fruits (Usall et al., 2000; Nunes et al., 1998).

Infection of fruits by post-harvest pathogens can occur in the field prior harvest, thus it may be advantageous to treat crops before harvest, which would reduce initial infection and then remain active and suppress pathogen infections during and after the harvest phase. Most of the biocontrol agents have been applied in post-harvest, few reports examined pre-harvest application for postharvest control of pome fruit (Leibinger et al., 1997; Teixidó et al., 1999). Also with pre-harvest application less fruit manipulations would be involved so that there would be less potential for damage through injuries that can occur during any postharvest treatment. Recent studies of combination of *C. sake* CPA-1 with *P. syringae* CPA-5 for controlling blue mold on pears and apples demonstrated good results. The concentrations of 5×10⁶ of *C. sake* and 2×10⁷ of *P. syringae* efficiently controlled blue mold at room and cold storage conditions (unpublished data).

The objectives of this work was to determine the efficacy of pre-harvest application of a combined treatment of *C. sake* CPA-1 and *P. syringae* CPA-5 to control *P. expansum* decay on pear and apple fruits during cold storage and study the population dynamics of each biocontrol agent.
Material and methods

Antagonists, pathogens, fruit and orchard
The antagonists used in this study were the yeast \textit{C. sake} CPA-1 and the bacterium \textit{P. syringae} CPA-5, both isolated from apple surface. Antagonist cells were prepared by growing cultures in nutrient yeast dextrose broth (NYDB). \textit{Penicillium expansum} was isolated from decayed apples after several months in storage and maintained on potato dextrose agar (PDA). ‘Golden Delicious’ apples and ‘Blanquilla’ pears were obtained from commercial orchards in Lleida, Catalonia.

Suppression of blue mold during cold storage by pre-harvest application
Twenty trees were selected at random and each treatment was repeated 4 times and each replicate consisted of 4 trees (85 fruits). Fruits were wounded (1 mm diameter and 2 mm deep) in two opposite positions in the equatorial zone 2 days before harvest. Pre-harvest treatments were applied 2 days before harvest using a handgun operating at 10 atmospheres pressure. Treatments were: \textit{C. sake} at 10^7 cfu/ml; \textit{P. syringae} 2×10^7 cfu/ml; mixed of antagonists at the same tested concentration in a 50:50 proportion. After harvest fruit were placed in separate boxes and a final inoculation of the pathogen by spraying the fruits with a 10^4 conidia/ml suspension of \textit{P. expansum} for 30 s. Once dried, the fruits were stored at 1°C and 90±5% RH in air. The number of infected wounds was determined after 4 months at cold storage. The test was repeated twice.

Population dynamics of mixed antagonists in wounded fruits
To determine population dynamics wounded and treated fruits from the assay above were sample at day 0 (just after treatment) and 2 (at harvest). After harvest fruits were placed on tray packs in plastic boxes and incubated at 1°C and 90±5% RH for 120 days. Twenty pieces of pear peel surface of 1.45 cm² and twenty-five pieces of apple peel surface of 2.5 cm² were removed with a cork bore from each fruit. Four fruits were selected for each replicate and each treatment was replicated four times. The peeled surface pieces of pears were shaken in a 50 ml and of apples in a 100 ml sterile phosphate buffer on a rotary shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasound bath. Ten-fold serial dilutions of the washings were made and plated on Petri dishes. Samples from populations of the antagonists alone or in mixture were plated in duplicate in NYDA supplemented with streptomycin to recover yeast cells and supplemented with imazalil (imazalil sulphate 99%) to recover bacteria cells. Colonies were counted after incubation at 25±1°C for 24 h for \textit{P. syringae} and 48 h for \textit{C. sake}. Population sizes were expressed as cfu/cm² of fruit surface. The experiment was carried out twice.

Results and discussion
The results of pre-harvest treatments to control postharvest blue mold in pears and apples are shown in Fig. 1. All treatments significantly reduced incidence of \textit{P. expansum} on pears and apples stored at 1°C for 4 months. On pears treated only with \textit{C. sake} CPA-1 or only with \textit{P. syringae} CPA-5, \textit{P. expansum} incidence was reduced to 53%. The combined treatment in pears was significantly different from the isolate application of each antagonist, reducing blue mold incidence in 90%, and enhanced biocontrol activity of each antagonist in 78% (Fig. 1A).

On ‘Golden Delicious’ apples an incidence reduction of 40% was observed with the application of \textit{P. syringae} CPA-5. Although no significant differences were observed between the individual applications of \textit{C. sake} CPA-2 or combined with \textit{P. syringae} CPA-5, a reduction of 46 and 56% respectively was observed. Regarding to the individual application
of *P. syringae* CPA-5, an enhancement of 26% of biocontrol activity was recorded with the combined treatment (Fig. 1B).

![Figure 1](image1.png)

**Figure 1.** Incidence of blue mold on A: ‘Blanquilla’ pear and B: ‘Golden Delicious’ apple treated in pre-harvest with *C. sake* CPA-1 (10^7 cfu/ml), *P. syringae* CPA-5 (2×10^7 cfu/ml), and their combination in a proportion of 50:50. Fruits were wounded and treated in the field two days before harvest. After harvest fruits were sprayed with *P. expansum* (10^4 conidia/ml) and stored at 1ºC and 90±5% RH for 120 days. Columns with the same letter are not significantly different (*P*≤0.05) according to the least significant difference test (LSD).

The population dynamics of *C. sake* CPA-1 and *P. syringae* CPA-5 are show in Fig. 2. At day 0 (day of treatment) and 2 (at harvest time) the population densities of both antagonists were higher in pears (Fig. 2A) than in apple (Fig. 2B). However at the end of the experiment, (day 120) population densities reached similar levels. In pears at day 0 and 2 the population level of *P. syringae* alone or in combination was higher than *C. sake* while in apple the opposite was observed at all sample moments.

![Figure 2](image2.png)

**Figure 2.** Population dynamics of *C. sake* CPA-1 and *P. syringae* CPA-5 in wounds of A: ‘Blanquilla’ pears and B: ‘Golden Delicious’ apple in day 0, 2 and 120. Fruits were wounded and treated in the filed 2 days before harvest and stored at 1º C and 90±5% RH for 120 days. Treatments were *C. sake* CPA-1 (10^7 cfu/ml), *P. syringae* CPA-5 (2×10^7 cfu/ml), and their combination in a proportion of 50:50. Symbols: ■ 100% CPA-1, □ 50% CPA-1, △ 100% CPA-5, ☐ 50% CPA-5.

This study demonstrate that the pre-harvest treatment with a mixture of 50:50 of *C. sake* CPA-1 and *P. syringae* CPA-5 enhanced biocontrol activity against *P. expansum* on apple and
pears in comparison with control by antagonists applied separately. Similar results were obtained in postharvest treatments using a mixture of *C. sake* CPA-1 and *Pantoea agglomerans* CPA-2 in apples and pears (Nunes et al., 2002). In that work similar population level of *C. sake* was obtained. The ability of both antagonists to colonize wounded fruits was not affected by the presence of the other antagonist, since similar level was obtained either alone or in combination. This result agrees with other authors (Janisiewicz & Bors, 1995) who concluded that the carrying capacity of the wounds is greater than the population of a single antagonist indicates. It could be possible that the enhancing effect of the mixture of both antagonists is due to the depletion of nutrients by their parallel growth in wounded fruits that does not allow the development of *P. expansum*.

In conclusion, our research showed that the pre-harvest application of a combination of *C. sake* CPA-1 and *P. syringae* CPA-5 results in an improvement of protection in comparison to the use of each antagonist alone.

**Acknowledgements**

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Increased biocontrol efficacy of *Brevibacillus brevis* against cucurbit powdery mildew by combination with neem extracts

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**Abstract:** This study has confirmed that the commercial neem extract, Trilogy, Certis, USA controls *Podosphaera xanthii* in several cucumber varieties. Furthermore, when the neem extract was combined with the bacterial biocontrol agent *Brevibacillus brevis* there was enhanced biocontrol. Indeed, effective control was still achieved when the concentration of Trilogy was reduced to only 0.05%. It has also been demonstrated that the neem extract operates by at least two modes action: directly by inhibiting *P. xanthii* conidia germination and indirectly, by inducing disease resistance in a systemic manner throughout the plant. Such combined use of BCAs has been advocated to broaden efficacy under different environments and to reduce the risk of resistance developing in the pathogen.

**Key words:** Azadirachta, *Podosphaera*, *Sphaerotheca*, Trilogy

**Introduction**

Powdery mildew infections caused by *Podosphaera xanthii* (formerly *Sphaerotheca fuliginea*) have led to considerable economic losses in agriculture. Like other diseases this has been implicated with the overuse of chemical fungicides resulting in risks of environmental pollution and the development of resistance in the pathogen (Seddon et al., 2000). Biological control offers an alternative to chemical control and many studies have been undertaken for powdery mildew. Concerns over the efficacy of biological control has led researchers to investigate the use of two or more agents and of particular relevance to powdery mildew is the use of bacterial biocontrol agents combined with plant extracts (Schmitt & Seddon, 2005). This research has investigated extracts from the neem tree and a bacterial biocontrol agent for disease control. The neem tree (*Azadirachta indica* A. Juss) and its extracts are well known for their use in traditional agriculture and medicine. The major limonoid, azadirachtin, has been advocated as a natural pesticide with much known about its efficacy and mode of action (Mordue (Luntz) et al., 2005). More recently researchers have shown interest in neem extracts for control of microbial pathogens including phytopathogens (Allan & Coventry, 2001). This study used clarified neem oil, supplied by Certis USA, and marketed as “Trilogy”. This product is reported to be moderately effective against powdery mildew of cucurbits although it may cause some phytotoxicity especially on repeated application. In addition, we tested the soil-inhabiting bacterium, *Brevibacillus brevis* that has been shown to have activity against a wide range of fungal pathogens including *Botrytis cinerea* (Seddon et al., 2000) and *P. xanthii* (Schmitt et al., 1999).

Combined use of two biocontrol agents (BCAs) with different modes of action has the objective of lowering the concentrations of the two BCAs used (thus lowering the environmental impact), so as to reduce the possibility of phytotoxicity and resistance development in the pathogen.
Materials and methods

Powdery mildew bioassay to test efficacy of biocontrol agents
The bioassay was developed by Dr. A. Schmitt (Institute for Biological Control, BBA, Germany). Cucumber seeds were grown in individual pots for 25-28 d, and the third leaf was excised. In most assays, the first two leaves were sprayed with the required concentrations of neem extract (Trilogy, Certis USA) and the third leaf was removed on the same day. Three days later, the plant was challenged with *P. xanthii* (1 × 10⁴ conidia ml⁻¹ in 0.0125% Tween 80) and if appropriate, with *B. brevis* a further one day later. All plants were incubated at 25±2°C (Fi-totron 600 H, England) with a 16 h photoperiod at 750-800 lx light intensity. Plants, not inoculated with pathogen or with BCAs were used appropriately as control treatments. Disease was scored by making a visual estimate of the % powdery mildew cover of the whole plant at daily intervals after the appearance of disease symptoms. *B. brevis* Nagano was cultivated in 200 ml Tryptic Soy Broth (TSB) contained in a 500 ml Erlenmeyer flask at 37°C at 150 rpm in darkness for 48 h (to obtain mostly vegetative cells) or 7 d (to obtain cultures consisting mainly of endospores). Cultures were diluted (1:3) with sterile distilled water (SDW) and applied to the upper surface of the leaves with an atomiser.

The effect of neem on *P. xanthii* conidial germination
Five microscope slides were sprayed with either SDW or 1% (v/v) neem extract solution and allowed to dry. Leaves from an infected cucumber plant were first shaken vigorously to remove old, non-viable spores and then were pressed to the slide surface to detach the conidia. A cover slip was then placed on each slide. All slides were incubated in a humid environment at 24°C and were assessed for germination of 200 conidia per slide 24 h later (Schmitt, pers. comm.). A subsequent experiment tested whether two applications of neem extract or water further affected germination since it is known that water can inhibit *P. xanthii* spore germination on the plant surface (Elad et al., 1996).

To determine whether neem extract induces resistance
As in the standard protocol, neem extract was applied to only the first true leaf on the day the third leaf was removed. This was done either once or repeated every 7 d. All the plants (n=5 per treatment), except the untreated controls, were sprayed with *P. xanthii* 3 d later. Scoring was undertaken as described earlier for both the whole plants and for each individual leaf.

Results and discussion

Initial experiments tested the ability of different doses of neem extract to suppress powdery mildew using the cultivar Knossos. Results showed that 1 and 2% neem extract (v/v), completely suppressed disease. At lower concentrations, disease symptoms were observed with a typical dose response. Thus, at 0.1% (v/v) neem extract, 30.1% leaf area was covered with disease symptoms, whereas plants treated with 0.05% (v/v) had 51.8%, plants treated with 0.025% (v/v) had 71.8% and plants treated with 0.001% had 81.6% leaf area infected. Plants used as control (untreated) had 19.6% leaf area covered by powdery mildew indicating that secondary infection was occurring within the time course of the experiment. Results showed that treatment with only *P. xanthii* had significantly higher levels of disease than those treated with neem extract and the untreated plants. There was no significant difference between the treatment of *P. xanthii* and either 0.025% (v/v) and/or 0.001% (v/v) neem treatments (P≥0.001). Similar results were found for cucumber varieties Pepinex, Chinese Snake and Femspot showing that the neem extract operated in a dose response manner.

Analysis showed that the neem extract significantly reduced *P. xanthii* spore germination (Table 1). With one application it significantly reduced conidia germination by
62%. Two applications of distilled water or neem extract, as expected, reduced spore germination further but this was not significantly different to when one application was made.

Table 1. Number of conidia germinated (n=200) after one or two applications of sterile distilled water or 1% (v/v) neem extract (Trilogy)

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Treatment</th>
<th>1 (Trilogy and SDW applied once)</th>
<th>2 (Trilogy and SDW applied twice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>176.6(a)</td>
<td>67(b)</td>
<td>145.4(a)</td>
</tr>
<tr>
<td>Mean ± std. error</td>
<td>± 4.1</td>
<td>± 13.5</td>
<td>± 5.1</td>
</tr>
<tr>
<td>1% Trilogy</td>
<td>53(b)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values followed by same letter indicate no significant difference (\(P=0.05\) using Tukey HSD post hoc tests).

It was evident that application of neem extract, to only the first leaf, reduced the development of powdery mildew over the whole plant. By the end of the experiment, all the leaves of the pathogen challenged plants that received no neem extract were infected with 97.1% infection overall. For the plants that received neem extract the overall infection was significantly different with only 22.2% disease symptoms. The first leaf showed 41.6% infection compared to 100% on the first leaf of plants receiving no neem extract. Of most interest is the fact that the 2nd and subsequent leaves also all showed lower infection than corresponding leaves on the plants receiving no neem extract. When neem extract was applied every 7 days, the infection on the first leaf was much lower (5.4%), and the infection on subsequent leaves was also lower. For example, the 2nd leaf on plants receiving no neem extract showed 100% infection; this decreased to 71% on plants that received one application on neem extract on the first leaf, and reduced further to 46.2% on plants that received repeated applications of neem extract to the first leaf. Weekly applications of neem extract reduced levels of *P. xanthii* infection compared to a single application, but this was not statistically significant.

Table 2. Final % of leaf area infected (n=3) for Chinese Snake cucumber plants testing *B. brevis* singly (after 48 h and 7d cultivation), neem extract (Trilogy) singly and in combination with each other

<table>
<thead>
<tr>
<th>Inoculated with <em>P. xanthii</em></th>
<th>Control No BCAs</th>
<th>48h <em>B. brevis</em></th>
<th>7d <em>B. brevis</em></th>
<th>0.05% (v/v) Trilogy</th>
<th>48h <em>B. brevis</em> + 0.05% (v/v) Trilogy</th>
<th>7d <em>B. brevis</em> + 0.05% (v/v) Trilogy</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.0 ±1.2</td>
<td>100 ±0</td>
<td>63.7(a) ±0.9</td>
<td>61.3(a) ±0.7</td>
<td>78.0(a) ±1.2</td>
<td>37.0(b) ±1</td>
<td>26.0(b) ±1</td>
</tr>
</tbody>
</table>

Values followed by same letter indicate no significant difference (\(P=0.05\)).

In the experiments testing the efficacy of *B. brevis* and neem extract used singly and in combination, both agents were found to be compatible with each other (data not shown). In
these experiments neem was used at the low concentration of 0.05% and *B. brevis* was applied after 48 h and 7 d cultivation in TSB.

Table 2 shows that plants challenged with pathogen alone developed 100% infection with *P. xanthii* after 25 d. Disease was reduced by 22% when 0.05% (v/v) neem extract was applied, by 36% when 48 h-old *B. brevis* was applied and by 38% when 7 d-old *B. brevis* was applied. There was no significant difference between the two ages of *B. brevis*. When used in combination the two BCAs gave enhanced disease control with a 63% reduction in *P. xanthii* infection in plants treated with 0.05% (v/v) neem extract and 48 h-old *B. brevis* and a 74% reduction for 0.05% (v/v) neem extract with 7 d-old *B. brevis*. There was no significant difference between using the different ages of *B. brevis*.

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**References**


On farm evaluation of biological control potential of some native isolates of *Trichoderma asperellum* on *Phytophthora megakarya*, the causative agent of cacao black pod disease in Cameroon

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⁴ MARS Inc. /USDA- ARS, Alternate Crops Systems Lab. Beltsville MD, USA, e-mail: prakash.hebbar@effem.com;
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**Abstract:** Fungal isolates PR10, PR11, PR12, 659-7 identified as *Trichoderma asperellum*, were isolated from the forest natural reserve of Dja (Cameroon) and from farmers’ fields around Yaoundé (Cameroon). Necrotrophic mycoparasitism was observed for all these isolates. Pre-colonized plates with the cacao black pod disease causative agent *Phytophthora megakarya* (NKOMIII) failed to infect healthy cacao pods 24 hours after contact with *T. asperellum* (PR11) and 48 hours after contact with PR10, PR12, and 659-7. On farm efficacy of these isolates was further evaluated during two years on field trials conducted in farmers’ fields in two different locations within the cacao growing zone of Cameroon. The experimental plots were set up in selected cacao farms left untreated with chemical fungicides for many years, and on which the pathogen pressure was well established. Six treatments were considered: *T. asperellum* (PR10, PR11, PR12, 657-9), a metalaxyl based chemical fungicide (Metalaxyl+cuprous oxide) and a non-treated control. In addition to routine management practices (pruning, sanitary harvest), sprays of biocontrol candidates and the metalaxyl based fungicide were made every two weeks and data were collected every week on individual trees. Plots treated with *T. asperellum* (PR11) yielded most, with higher number of mature and healthy cacao pods.

**Introduction**

*Phytophthora megakarya* the causative agent of cacao black pod disease is present only in Central and West African cocoa producing countries, and is the most damaging of the cocoa diseases in this region. Annual losses due to this disease range from 30 to 90% of total production (Dakwa, 1987). To combat this disease, copper and metalaxyl-based fungicides are applied. Apart from being very expensive for many African farmers, heavy reliance on such chemicals can be associated with non-target effects, loss of biodiversity and spoilage of land and water and also may lead to the development of resistance by the pathogen (Fontem et
al., 2005). As an alternative to chemical fungicides application, agronomic measures such as the removal of diseased pods and shade management have not proven effective enough (Tondje et al., 1993; Ndoumbe-Nkeng et al., 2004). Moreover, a cacao variety resistant to black pod disease caused by P. megakarya is yet to be made available.

The biological control of plant diseases has become a research area in view of reducing the hazardous impact of chemical pesticides. There are very few reports on the use of microbial control on P. megakarya the most damaging causative agent of cacao black pod disease in the world, only present in Central and West Africa. The biological control strategy by mass production and release in cacao plantations of one or more natural microbial antagonists is expected to offer an environmentally friendly alternative to combat P. megakarya black pod disease. The aim of the research reported in this paper was to isolate mycoparasites against P. megakarya and identify candidates that could be developed into effective biological control of this pathogen under field condition.

Materials and methods

**Fungal isolates**

Isolates of *Trichoderma* spp., were isolated as follows: In a beaker containing 50 ml of sterilized distilled water, 10 g of soil sample collected from upper 8 cm of organic soil from natural forest reserves or annual crop fields within the forest zone of Cameroon were mixed and stirred for 10 min and 1ml of the mixture was pipetted onto the surface of each of 3 plates containing modified Potato dextrose Agar medium with 0.015% rose Bengal and 0.025% chloramphenicol, or onto a *Trichoderma* selective medium (TME) (Papavizas, 1982). And spores were mass produced by solid state fermentation on rice as substrate. Conidia were harvested and calibrated at 10^8 CFU/g of a formulation with sterile cassava flour.

*P. megakarya* Strain NKOM3-00 was isolated from a naturally infected cacao pod in a farmer’s field at Nkometou III and stored in sterile distilled water (Jones et al., 1991) on small pieces of V8 agar (200 g V8 juice, 3 g CaCO₃, 15 g agar and 1000 ml distilled water).

**Identification and characterization of fungal isolates**

Four *Trichoderma* isolates that showed promise as mycoparasites of *P. megakarya* (659-7, PR10, PR11, PR12) were identified based on morphological features following Samuels et al. (1999) as *T. asperellum*. *T. asperellum* is notable for having subglobose to ovoidal, finely spinulose conidia produced on relatively long phialides from conidiophores that prominently show paired branches (http://nt.ars-grin.gov/taxadesccriptions/keys/TrichodermaIndex.cfm). This species does not grow at 37°C. *T. asperellum* isolates 659-7, PR10, PR11, and PR12 were further characterized by ITS-RFLP analysis, translation elongation factor 1-alpha (EF-1α) sequencing analysis, and RAPD-PCR analysis.

**In vitro tests**

Mycoparasites were isolated using the pre-colonized plate method (Foley & Deacon, 1985; Krauss et al., 1998). In our case we used discs (5 mm diameter) of sterile filter paper: Sterile filter papers were individually deposited onto growing mycelia of a young culture of fungus. Four days later the sterile filter paper was covered by the mycelia. Each of these covered filter papers where then carefully removed and carefully transferred on top of mycelium of a ten days old culture of *P. megakarya*. Growth of the tested biocontrol candidate on *P. megakarya* was then observed daily for three weeks.

In case of observed mycoparasitism on pre-colonized plates, each strain with effective mycoparasitism and *P. megakarya* were sub cultured on separate plates and incubated until fully and uniformly colonized. A strip of agar with mycoparasite mycelium (0.5×5 cm) was excised and carefully placed up-side-down on to the growing mycelium of *P. megakarya*.
Plates were incubated for 24 hours to allow the mycoparasite to attach to *P. megakarya* mycelium, and inverted to avoid contamination due to water condensation (Krauss et al., 1999) (Fig. 1ab).

**Field trial**

We report here field trials that were carried out between 2002 at Metet (near Yaoundé) and in 2003 at Nkoemvone (15 km from Ebololowa). The experimental plots were set up within a completely randomized bloc’s experimental design with replicates in cacao fields left untreated with chemical fungicides since many years, and on which the pathogen pressure was well established. 6 treatment were evaluated (four selected strains of *Trichoderma asperellum* (PR10, PR11, PR12, 657-9), a chemical fungicide (Ridomil plus: 120 g metalaxyl + 600 g cuprous oxide /kg) and a non treated control) within bloc, an elementary plot was composed of 25 cacao trees with 4 replicates at Nkoemvone, and 20 trees per treatment at Esse in 2002. Routine management practices were maintained in the field before and throughout the experimentation. *T. asperellum* (PR10, PR11, PR12, 657-9) and the chemical fungicide (Ridomil plus: 120 g metalaxyl + 600 g cuprous oxide/kg) were applied at 14-day interval. Two applications of each treatment were made during the experiments. All the treatments were applied in liquid suspension (100 g formulation at 10⁸ CFU/g in a 15 L tank) using a knapsack sprayer and 150 ml/tree were applied in one pass over each treatment. Disease ratings were taken for each tree in each plot every week after the first application till the end of the trial.

**Data analysis**

Statistical analyses were conducted using SAS/STAT software v8.2 for IBM-PC (SAS Institute Inc. 2001). And Significance was evaluated at *P*<0.05 for all tests. An analysis of deviance according to the GENMOD procedure (SAS Institute Inc 2001) was done separately for each measured variable. Significance was evaluated at *P*<0.05 for all tests. Comparisons of means were done using the method of contrasts.
Results

**In vitro tests**
Speed of necrotrophic mycoparasitism (Fig. 2) was higher for PR11: (4 days after, *P. megakarya* was suppressed 40 mm away from initial point of contact between *P. megakarya* and *T. asperellum* PR11) followed by PR12 (30 mm after 4 days). Six days after, PR11, PR12, and 659-7 had already suppressed *P. megakarya* 50 mm away from initial contact point between *T. asperellum* (PR11) and *P. megakarya* (NKOMIII), and 8 days after, *T. asperellum* PR10 suppressed *P. megakarya* on plate 50 mm away from the point of initial mycoparasitism (Table 2).

![Graphs showing distance vs time for different mycoparasitic strains](image)

**Figure 2.** Speed of Necrotrophic mycoparasitism assessment on precolonized plates

**Field experiments**
There is an increase of disease occurrence in all the biocontrol candidates during increased rains. However, PR11 has the more consistent results and has the lowers disease scores of the selected biocontrol candidates.
Table 1. Cumulative total mature pods, total pods, and percent diseased pods per cacao tree for *T. asperellum* treatments in a short-term field trial conducted in 2002 near Esse, Cameroon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total pods (no/tree)</th>
<th>Healthy pods (no/tree)</th>
<th>Total diseased pods (no/tree)</th>
<th>Diseased pods (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. asperellum</em> 657-9</td>
<td>15.8 c</td>
<td>12.8 b</td>
<td>2.8 c</td>
<td>17.7 c</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR10</td>
<td>19.2 b</td>
<td>16.7 a</td>
<td>2.4 c</td>
<td>12.5 c</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR11</td>
<td>17.2 c</td>
<td>16.7 a</td>
<td>0.3 a</td>
<td>2.0 a</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR12</td>
<td>22.1 a</td>
<td>18.7 a</td>
<td>3.3 c</td>
<td>14.9 c</td>
</tr>
<tr>
<td>Ridomil</td>
<td>18.9 b</td>
<td>16.8 a</td>
<td>1.4 b</td>
<td>7.4 b</td>
</tr>
<tr>
<td>Control</td>
<td>16.8 c</td>
<td>11.1 b</td>
<td>4.9 d</td>
<td>29.1 d</td>
</tr>
</tbody>
</table>

* Analysis values followed by the same letter are not significantly different at *P*<0.05.

Table 2. Cumulative total mature pods, total pods, and percent diseased pods per tree for *T. asperellum* treatments in a long-term field trial conducted near Nkoemvone, Cameroon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total pods (no/tree)</th>
<th>Healthy pods (no/tree)</th>
<th>Diseased pods (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>July – August</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. asperellum</em> 659-7</td>
<td>15.9 b</td>
<td>15.2 e</td>
<td>4.2 a</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR10</td>
<td>15.5 b</td>
<td>13.9 d</td>
<td>9.4 b</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR11</td>
<td>13.6 b</td>
<td>11.9 c</td>
<td>10.4 b</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR12</td>
<td>15.0 b</td>
<td>12.7 c</td>
<td>13.9 c</td>
</tr>
<tr>
<td>Ridomil Plus</td>
<td>11.5 a</td>
<td>10.5 a</td>
<td>7.7 b</td>
</tr>
<tr>
<td>Water control</td>
<td>11.8 a</td>
<td>9.6 b</td>
<td>19.3 d</td>
</tr>
<tr>
<td>September</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. asperellum</em> 659-7</td>
<td>19.1 b</td>
<td>11.6 b</td>
<td>39.2 b</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR10</td>
<td>18.5 b</td>
<td>7.4 c</td>
<td>58.1 c</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR11</td>
<td>19.1 b</td>
<td>11.8 b</td>
<td>37.5 b</td>
</tr>
<tr>
<td><em>T. asperellum</em> PR12</td>
<td>15.3 a</td>
<td>6.6 c</td>
<td>55.7 c</td>
</tr>
<tr>
<td>Ridomil Plus</td>
<td>18.9 b</td>
<td>15.8 a</td>
<td>15.9 a</td>
</tr>
<tr>
<td>Water control</td>
<td>19.5 b</td>
<td>7.8 c</td>
<td>60.0 c</td>
</tr>
</tbody>
</table>

* Analysis of data collected weekly at Nkoemvone revealed 3 periods: July–August, low diseased pod losses; September, greatest pod losses; and October–November, number of pods/tree was low in many treatments due to large diseased pod losses in September and high number of ripened pods (data not shown). The data was subdivided into three portions (July–August, September, October–November) prior to analysis. Mean values followed by the same letter are not significantly different at *P*<0.05.

**Discussion**

The tested strains of *T. asperellum* showed high necrotrophic mycoparasitism on *P. megakarya* strain NKOMIII. However PR11 and PR12 expressed necrotrophic mycoparasitism more earlier on the cacao black pod disease pathogen. On the potential biological control agents tested in this study, *T. asperellum* (PR11) has most consistently shown potential as biological control agent by reducing the number of diseased pods on treated cacao trees. This is the first report on effective microbial control of *P. megakarya* with *Trichoderma* sp. in field conditions.

*Trichoderma* sp. are ubiquitous soil saprophytes (Papavizas, 1985). They have been found to be effective biological control agents against a large number of plant pathogenic
fungi in various crops. The main mechanism of action of this species is necrotrophic mycoparasitism (Mukhopadhyay and Mukherjee, 1996). By virtue of being soil saprophytes Trichoderma sp are good competitors (Harman & Bjorkman, 1998). Trichoderma sp. are highly diverse and ecologically successful fungi. Recently, these fungi have been used in quite significant amounts in commercial agriculture (about 30% of the total soil fungicide used in the greenhouse industry in the USA are products based on T. harzianum strain T22). Until recently, Trichoderma sp. were believed to achieve biocontrol by direct effects on fungal pathogens, particularly via mycoparasitism, antibiosis and competition only. While these mechanisms are important, direct effects on plants are increasingly found to be equally important (Harman, 2000).

The formulation of T. asperellum has shown to be very important in affecting its biocontrol ability: we have observed wash off of the formulation and increased disease levels attacks on pods during constant heavy rains. More investigation on waterproof formulation might have to be pursued, coupled with studies on methods of application for a better efficacy of these biological control candidates. Integration of biological control to other controls needs to be investigated for better integration disease management.

References


Bacillus subtilis strain QST 713, use in integrated pest management

Donald W. Edgecomb, Denise Manker
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Abstract: Bacillus subtilis QST 713, a naturally occurring bacterial strain, was discovered in a California orchard by AgraQuest, Inc., USA. B. subtilis QST 713 has been shown to possess significant efficacy against a broad spectrum of bacterial and fungal pathogens and is not toxic to beneficial and non-target organisms. As determined by US-Environmental Protection Agency and international regulatory authorities, B. subtilis QST 713 is exempt from the requirement of a tolerance because there are no synthetic chemical residues, and it is safe to workers and the environment. As a result, treated fruit and vegetables can be exported throughout the world without restrictions. B. subtilis QST 713 works through multiple modes of action that involve the competitive biological action of B. subtilis in addition to fungicidal, lipopeptide compounds produced by the bacterium, including novel molecules. B. subtilis QST 713 has been shown to be an effective tool for disease control in organic crop production and in integrated disease control programs contributing to resistance management and, overall, reducing dependency on synthetic fungicides.

Description and registration status

Bacillus subtilis is a rod-shaped, gram positive, aerobic, motile bacterium which is ubiquitous in nature. The bacterium can also produce an endospore. B. subtilis is commonly found in various ecological niches including soil, water and air. The US-Environmental Protection Agency and international regulatory authorities have classified B. subtilis QST 713 as a microbial fungicide. The commercial formulated product and QST 713 technical product contain living B. subtilis strain QST 713 as the active ingredient. B. subtilis QST 713 was approved in the United States as a foliar fungicide in 2000 and is currently registered in 16 countries under the trade name, Serenade® (Table 1).

<table>
<thead>
<tr>
<th>Year</th>
<th>Chile</th>
<th>US</th>
<th>Mexico</th>
<th>Costa Rica</th>
<th>Japan</th>
<th>Guatemala</th>
<th>France</th>
<th>Columbia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Deutschland</td>
</tr>
<tr>
<td>2001</td>
<td>Chile</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Germany</td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thailand</td>
</tr>
<tr>
<td>2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spain</td>
</tr>
<tr>
<td>2004</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Greece</td>
</tr>
<tr>
<td>2005</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Africa</td>
</tr>
<tr>
<td>2006&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Australia</td>
</tr>
</tbody>
</table>

All 1999-2005 were regulatory approved for commercial use.
Crop use and disease spectrum

*Bacillus subtilis* QST 713 efficacy has been demonstrated on over 30 crops in 20 countries against a broad spectrum of fungal and bacterial pathogens. Currently, the major commercial uses are in the crops listed in Table 2. *B. subtilis* QST 713 can be applied alone, in tank mixes or in rotation programs with other fungicides. Therefore, it is ideally suited for “high” value, fruit and vegetable production particularly for export markets with MRL restrictions, requirements for organic or reduced chemical-input certification.

Table 2. *Bacillus subtilis* QST 713: Global commercial use

<table>
<thead>
<tr>
<th>Crop</th>
<th>Disease</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato/Pepper</td>
<td>Bacterial Leaf Spot</td>
<td><em>Xanthomonas</em> spp.</td>
</tr>
<tr>
<td></td>
<td>Powdery Mildew</td>
<td><em>Leveillula taurica</em></td>
</tr>
<tr>
<td></td>
<td>Early Blight</td>
<td><em>Alternaria solani</em></td>
</tr>
<tr>
<td>Grapes</td>
<td>Gray Mold</td>
<td><em>Botrytis cinerea</em></td>
</tr>
<tr>
<td></td>
<td>Powdery Mildew</td>
<td><em>Uncinula necator</em></td>
</tr>
<tr>
<td></td>
<td>Sour Rot</td>
<td>Multiple pathogens</td>
</tr>
<tr>
<td>Cucurbits</td>
<td>Powdery mildew</td>
<td><em>Erysibe/Sphaerotheca</em> spp.</td>
</tr>
<tr>
<td></td>
<td>Gummy Stem Blight</td>
<td><em>Didymella bryoniae</em></td>
</tr>
<tr>
<td>Banana</td>
<td>Black Sigatoka</td>
<td><em>Mycosphaerella fijiensis</em></td>
</tr>
<tr>
<td>Mango</td>
<td>Anthracnose</td>
<td><em>Colletotrichum gloeosporioides</em></td>
</tr>
<tr>
<td>Lettuce</td>
<td>Leaf Drop</td>
<td><em>Sclerotinia</em> spp.</td>
</tr>
<tr>
<td>Apples/Pears</td>
<td>Fire Blight</td>
<td><em>Erwinia amylovora</em></td>
</tr>
<tr>
<td>Beans</td>
<td>White Mold</td>
<td><em>Sclerotinia sclerotiorum</em></td>
</tr>
</tbody>
</table>

Modes of action

*Bacillus subtilis* QST 713 works through several modes of action that are manifested by the bacterium colonizing the leaf surface and competing with the pathogen for nutrients and space and physically preventing attachment and penetration of the pathogen. In addition, *B. subtilis* QST 713 produces three groups of metabolites known as lipopeptides, (iturins, agrastatins/plipastatins, and surfactins) that act in a synergistic manner to destroy pathogen germ tubes and pathogen membranes. The iturins and plipastatins have been reported to have fungicidal activity. *B. subtilis* QST 713 is the first strain reported to produce iturins, plipastatins and surfactins and two new compounds, the agrastatins. Studies at AgraQuest on the effects of the individual groups of lipopeptides on pathogen spore germination compared to mixtures of the groups, provided a better understanding of the role of these metabolites. Morphological differences were observed in spores treated with different lipopeptide groups. The iturin group resulted in inhibition of spore germination and was dependent upon the concentration of iturins present. The iturins were most effective on *Botrytis cinerea* conidia with an EC_{50} as low as 15 ppm (50% inhibition of spore germination). The EC_{50} of *Monilinia fructicola* conidia occurred at 30 ppm and for *Alternaria brassicicola* the level required was 25 ppm. Exposure of the spores to the iturin/plipastatin group resulted in an abnormal appearance in which the spore had a large bubble-like growth replacing the normal appresorium. This effect was most notable with *A. brassicicola* spores in which the EC_{50} was 5 ppm. Investigation of the effects of combining the groups of lipopeptides gave further
explanation for the efficacy observed with *B. subtilis* QST 713. Addition of concentrations as low as 1 ppm agrastatin/plipastatin to 10 ppm iturin provided a significant reduction in spore germination; reduced to approximately 5% conidia germination for *M. fructicola*. The surfactin group was found to have no effect on spores at the highest rate tested (250 ppm). Addition of 25 ppm surfactin to 20 ppm iturin reduced spore germination from 85% to less than 5%. Surfactin at 25 ppm added to agrastatin/plipastatin reduced germination from 100 to 10%. Given the novel, multiple, modes of action, *B. subtilis* QST 713 is utilized as a resistance management tool in rotation programs with chemical fungicides, such as the strobilurin and triazole groups, which are highly susceptible to resistance development due to a specific metabolic site, modes of action.

**Formulation**

*Bacillus subtilis* QST 713 is formulated as a wettable granule and aqueous suspension product containing from 1 to $7 \times 10^9$ cfu/gram depending upon the formulation. It can be applied in conventional application equipment, requires no special storage conditions and has a shelf life of more than 2 years. *B. subtilis* QST 713 formulations have been shown to be compatible in mixtures with commonly used synthetic fungicides (e.g., sulfur, copper hydroxide, mancozeb, chlorothalonil, azoxyxystrobin, myclobutanil) and are approved for use in organic agriculture under the guidelines established by the Organic Materials Review Institute (OMRI-USA), Institute for Marketecology (IMO-Switzerland) and BCS Öko-Garantie (BCS-Germany).

**Use in integrated disease management programs**

Because of the excellent environmental profile, broad disease control spectrum and safety to non-target, beneficial organisms (Table 3), *Bacillus subtilis* QST 713 is ideally suited for use in integrated pest management (IPM) programs that utilize many approaches such as cultural practices, classical biological control and other fungicides.

Table 3. Summary of *Bacillus subtilis* strain QST 713 ecological toxicity studies

<table>
<thead>
<tr>
<th>Non Target Test Organism</th>
<th>Toxicity</th>
<th>Toxicity Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian oral (quail)</td>
<td>LD50 &gt; 5000 mg/kg</td>
<td>5</td>
</tr>
<tr>
<td>Freshwater fish (trout)</td>
<td>LC50 = 162 ppm</td>
<td>3</td>
</tr>
<tr>
<td>Honey bee larvae</td>
<td>LC50 &gt; 10,000 ppm</td>
<td>5</td>
</tr>
<tr>
<td>Daphnia</td>
<td>EC50 = 108 ppm</td>
<td>3</td>
</tr>
<tr>
<td>Hymenoptera parasitic wasp</td>
<td>LC50 &gt; 30,000 ppm</td>
<td>5</td>
</tr>
<tr>
<td>Lady beetle</td>
<td>LC50 &gt; 60,000 ppm</td>
<td>5</td>
</tr>
<tr>
<td>Lacewing larvae</td>
<td>LC50 &gt; 60,000 ppm</td>
<td>5</td>
</tr>
</tbody>
</table>

1 Rating according to Hodge and Sterner scale of 1 to 6 where 1 is “Extremely Toxic” and 6 is “Relatively Harmless”

The benefits of *B. subtilis* QST 713 in programs utilizing chemical fungicides are shown in the following studies. The objective of these studies were (1) to demonstrate the efficacy of *B. subtilis* QST 713 used alone against economically important diseases and (2) to document the effectiveness of *B. subtilis* QST 713 in strategically replacing conventional fungicide applications in rotation and tank mix programs. In a 2003 cucumber study conducted in Japan,
B. subtilis QST 713 provided effective powdery mildew (Sphaerotheca fuliginea) control when applied alone on a weekly schedule, although it was slightly less effective than the chemical standard, quinomethionate (Table 4). However, rotational programs with B. subtilis QST 713 and quinomethionate provided excellent powdery mildew control, comparable to weekly applications of quinomethionate alone. The B. subtilis QST 713/quinomethionate programs provided a 50% reduction in chemical fungicide use without compromising disease control.

B. subtilis QST 713 is also effective in tank mix programs with reduced use rates of chemical fungicides as shown in a banana/black sigatoka (Mycosphaerella fijiensis) study conducted in Costa Rica during 2003 (Table 5). Black sigatoka causes significant reductions in functional leaf area resulting in yield losses and premature ripening of harvested fruit. Successful sigatoka control programs depend heavily on synthetic fungicides. As many as 30 to 50 fungicide applications per year are required for acceptable sigatoka control creating concerns for adverse affects on local ecosystems and potential health risks associated with human exposure. Systemic fungicides, such as strobilurin, sterol biosynthesis and sterol demethylation inhibitors, provide effective sigatoka control, but are highly susceptible to disease resistance development if not properly managed. In this study, evaluations parameters such as Youngest Leaf Infected and Functional Leaves at Shooting and Harvest showed that B. subtilis QST 713 provided effective control of sigatoka, particularly in tank mix combinations, with reduced rates of the standard protectant fungicide, mancozeb.

Table 4. Bacillus subtilis QST 713 integrated pest management program for cucurbit powdery mildew (Sphaerotheca fuliginea) control, Japan 2003. Chemical fungicide = quinomethionate.

<table>
<thead>
<tr>
<th>Treatment Schedule</th>
<th>Powdery Mildew Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Water</td>
</tr>
<tr>
<td>Chemical</td>
<td>Fungicide</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>B. subtilis</td>
</tr>
<tr>
<td>Chemical / B. subtilis</td>
<td>Fungicide / B. subtilis</td>
</tr>
<tr>
<td>B. subtilis / Chemical</td>
<td>Fungicide / B. subtilis</td>
</tr>
</tbody>
</table>

Table 5. Bacillus subtilis QST 713 integrated pest management program for black sigatoka (Mycosphaerella fijiensis) control in bananas, standard fungicide = mancozeb 60 OS formulation. Mindanao Philippines (March to August 2003)

<table>
<thead>
<tr>
<th>Treatment (rate / hectare)</th>
<th>Visible Streaks</th>
<th>Youngest Leaf Spotted</th>
<th>Functional Leaves at Shooting Stage</th>
<th>Functional Leaves at Harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis – 2 liters</td>
<td>3.9 a</td>
<td>9.5 c</td>
<td>13.3 a</td>
<td>7.8 a</td>
</tr>
<tr>
<td>B. subtilis + mancozeb (2 + 0.9 liters)</td>
<td>3.9 a</td>
<td>10.1 bc</td>
<td>13.5 a</td>
<td>7.4 a</td>
</tr>
<tr>
<td>Standard Program (mancozeb at 1.8 liters)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>3.4 b</td>
<td>8.6 d</td>
<td>12.9 a</td>
<td>4.9 b</td>
</tr>
</tbody>
</table>

B. subtilis QST 713 has been shown to be an effective tool in rotational and tank mix programs with other fungicides contributing to resistance management and overall, reducing dependency on synthetic fungicides.
Combination of microbial biocontrol agents to control rhizoctonia damping-off and fusarium wilt of tomato

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² Department of Genetic, Breeding and Biotechnology, Research Institute of Vegetable Crops, Konstytucji 3-Maja 1/3, 96-100 Skierniewice, Poland, e-mail: bdyki@inwarz.skierniewice.pl

Abstract: Mixtures of bacteria with different modes of action were used to control Rhizoctonia damping-off and Fusarium wilt of tomato plants. In the experiments with R. solani combined bacteria reduced variability of biocontrol exhibited by single strains of these bacteria and the effect of the mixtures was more stable. Application combined bacteria to the roots of tomato transplants also significantly enhanced suppression of Fusarium wilt.

Key words: bacteria mixture

Introduction

In recent decades numerous bacteria have been identified to be able to suppress plant pathogens (Weller, 1988; Shoda, 2000). However, only a few of these agents have been commercialized, because inoculation of soil with single strain of biocontrol agent rarely lead to a satisfied level of plant protection, and positive effect was often inconsistent. The way to improve their efficacy may consist in using combinations of the agents, especially when they exhibit different modes of action. In the environment, where natural conditions are variable, one mechanism may compensate another resulting in an additive and more stable effect of biocontrol. This thesis is supported by de Boer et al. (1999), Rapauch & Kloeppep (1998) or Szczech & Shoda (2004), who demonstrated that the mixture of active microorganisms provided better plant protection than the use of single strain.

In our studies various combinations of active bacteria (MIX) were examined for their efficacy in control of Rhizoctonia solani (RS) and Fusarium oxysporum f. sp. lycopersici (FOL) on tomato plants. The effect of separate strains and their combination on the population of Fusarium sp. in the rhizosphere of treated tomato plants was also studied.

Material and methods

Bacterial strains
The bacteria used in the experiments were isolated from peat mixed with vermicompost and from the rhizosphere of vegetable plants (tomato, lettuce, cucumber). The bacteria were preliminary identified as pseudomonads, except the strain T20. They exhibited various mechanisms of biocontrol: antagonism, plant growth promotion and induced resistance (Table 1). Inoculation of tomato roots with single strains 125, 207 and PT60 significantly stimulated development of lateral roots, especially in plants grown in the medium infested with FOL (data not presented). In general bacteria used to prepare the mixtures were compatible, and
only strains PT42 and SZ141 slightly inhibited strains PT60 and T20 in \textit{in vitro} tests. The effect of combinations of these bacteria in control of rhizoctonia damping-off and fusarium wilt was studied in a growth-chamber and greenhouse experiments respectively.

Table 1. Characteristic of biocontrol activity of bacterial strains used to prepare the mixtures

<table>
<thead>
<tr>
<th>Effects</th>
<th>Bacterial strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125</td>
</tr>
<tr>
<td>Plant growth stimulation</td>
<td>+</td>
</tr>
<tr>
<td>Development of root system</td>
<td>+</td>
</tr>
<tr>
<td>Antagonism against</td>
<td>–</td>
</tr>
<tr>
<td>\textit{R. solani}</td>
<td>–</td>
</tr>
<tr>
<td>\textit{F. oxysporum}</td>
<td>–</td>
</tr>
<tr>
<td>Induction of resistance</td>
<td>–</td>
</tr>
</tbody>
</table>

”+” positive effect, ”–“ no effect, ”nd“ not studied.

\textbf{Bacterial mixtures in control of rhizoctonia damping-off}

Tomato seeds cv. Remiz were soaked for 15 min in cell suspension of single or mixed bacterial strains 125, 207, PT42 and SZ141. First mixture (MIX I) contained strains 125, 207 and PT42, and second mixture (MIX II) contained strains PT42 and SZ141. Control seeds were soaked in 0.85% solution of NaCl. To prepare the mixtures equal volumes of "single" bacterial suspensions were combined just before application. The density of cells in the suspension was about $10^{10}$ cfu ml\(^{-1}\). Treated seeds were planted in the potting medium infested with RS in plastic "mini-chamber" system (Tigret, Poland). One mini-chamber contained 50 g of infested medium planted with 10 seeds. For each treatment three replications were prepared and tests were repeated three times. Planted mini-chambers were incubated for 7 days in a growth chamber and the number of growing tomato seedlings was estimated.

\textbf{Bacterial mixtures in control of fusarium wilt of tomato}

In the experiment with FOL the roots of tomato plants cv. Remiz were dipped in bacterial suspensions. Water suspensions of single strains PT42, SZ141, PT60, T20 and three mixtures of these bacteria were used: PT42+SZ141+T20 (MIX III), PT42+PT60+T20 (MIX IV), PT60+SZ141+T20 (MIX V). To prepare the mixtures, equal volumes of "single" bacterial suspensions were combined just before application. The density of cells in the suspension was about $10^{9}$ cfu ml\(^{-1}\). Washed roots were kept in the suspensions for 15 min and then the plants were planted into pots containing medium infested with FOL. Each treatment was prepared in three replications. All tests were repeated twice. Plants were grown in a greenhouse for one month and then the severity of wilt was estimated according to a 0-4 disease index: 0-no disease symptoms; 4-100% necrosis of the stem base cross section. The density of \textit{Fusarium} spp. in the rhizosphere of treated tomato was estimated on Komada's medium (Dhingra & Sinclair, 1995).

\textbf{Results and discussion}

Treating of tomato seeds and roots with the mixtures of selected bacterial strains resulted in decrease of rhizoctonia damping-off and fusarium wilt. In the experiment with RS, the effect of the mixtures was not always better than the effect of single strains (Fig. 1). However, the
level of protection obtained by separate strains was variable in consecutive tests, while the
efficacy of bacterial mixtures was more stable. The number of emerged seeds treated with the
mixtures was always higher than the number of seedlings grown from seeds not treated with
bacteria.

![Figure 1. Effect of bacterial mixtures MIX I (A-C) and MIX II (D-F) on rhizoctonia damping-off of
tomato plants in three tests. Data were subjected to variance analysis and t-test was used to estimate
the significance of differences between means.](image)

Application of different combinations of antagonistic strains of bacteria PT42, SZ141,
PT60 and T20 to the roots of tomato transplants resulted in a significant protection against
fusarium wilt (Table 2). Although, the strains PT42 and SZ141 were not compatible in in
vitro tests with PT60 and T20, the mixtures of these strains were more effective than bacteria
used separately. The biocontrol effect was consistent in two consecutive tests. Strain T20 used
separately did not protect plants (Table 2). However, when it was removed from the mixtures
their protective effect was decreased (data not presented). It suggests that T20 although
incompatible might stimulate the activity of other bacterial components of the mixtures. PT60
and T20 were not effective in control of RS (data not presented), but PT42 and SZ141 were
able to suppress both pathogens. PT42 and SZ141 decreased also density of fusarium in the
rhizosphere of treated plants. However, the reduction of the pathogen by the mixtures was not
significant (Table 2) and the effect was most likely the result of the different mechanisms of
bacterial activity. Enhanced disease suppression and reduced variability of the control, when
the combinations of active microorganisms were used, obtained also Rapauch & Kloeppper
(1998), Guetski et al. (2001) and de Boer et al. (2003).
Table 2. The effect of bacterial mixtures on the wilt and the density of *Fusarium* spp. in the rhizosphere of tomato plants

<table>
<thead>
<tr>
<th></th>
<th>Exp. FOL</th>
<th>PT42</th>
<th>SZ141</th>
<th>PT60</th>
<th>T20</th>
<th>MIX III</th>
<th>MIX IV</th>
<th>MIX V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease index: 0 - 4</td>
<td>1</td>
<td>2.0 a</td>
<td>1.5 a</td>
<td>1.8 a</td>
<td>–</td>
<td>2.8 a</td>
<td>1.2 a</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
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<td>1.8 b</td>
<td>–</td>
<td>2.3 a</td>
<td>2.5 a</td>
<td>1.4 bc</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>2.6 ab</td>
<td>–</td>
<td>1.4 cd</td>
<td>2.1 bc</td>
<td>3.2 a</td>
<td>–</td>
<td>–</td>
<td>0.8 d</td>
</tr>
</tbody>
</table>

*Fusarium* density in the rhizosphere

<table>
<thead>
<tr>
<th></th>
<th>Exp. FOL</th>
<th>PT42</th>
<th>SZ141</th>
<th>PT60</th>
<th>T20</th>
<th>MIX III</th>
<th>MIX IV</th>
<th>MIX V</th>
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<tr>
<td>1</td>
<td>6.9</td>
<td>4.8</td>
<td>4.2</td>
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<td>5.3</td>
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<td>3.1</td>
<td>–</td>
<td>4.8</td>
<td>4.9</td>
<td>–</td>
<td>2.4</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>6.9</td>
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<td>3.5</td>
<td>6.3</td>
<td>4.1</td>
<td>–</td>
<td>–</td>
<td>3.3</td>
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</tbody>
</table>

Data represent disease index are the averages of two experiments. They were subjected to analysis of variance and *t*-test was used to estimate the significance of differences between the means in rows. There were not significant differences in *Fusarium* density between treatments.

References


Sensitivity to fungicides of wild and mutant strains of *Trichoderma* spp. for integrated control of tomato root and crown rot

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**Abstract:** *In vitro* sensibility of mycelia and conidia of *Trichoderma* strains to fungicides was studied. *Trichoderma* wild strains of several species (*T. harzianum*: Th 650, Th V, Th 291, Th 11, Th12; *T. viride*: Tvir 32; *T. piluliferum*: Tpi 33, and *T. polisporum*: Tpo 34) previously characterized and selected as good bioantagonists to control tomato root and crown rot (*Fusarium oxysporum* f. sp. *lycopersici*, *F. solani*, *Pyrenochaeta lycopersici*, *Phytophthora nicotianae*, and *Rhizoctonia solani*) were tested. Mutants were obtained with nitrosoguanidine and UV light, and their biocontrol activity was assessed on the pathogens mentioned above. The fungicides evaluated which are used normally or should be used to control tomato root and crown root diseases, were Aliette, Enzone, Hymexazol, Metalaxil, Mertec, Metalaxil, Monceren, Phyto-Fos, Rovcap and Rovral, The *Trichoderma* wild strains ThV, Tpi 33 and Th 11, had higher EAC₅₀ at least for two fungicides, being less sensitive to the agrochemicals evaluated than the other wild strains. Fungicides Hymexazol and Phyto Fos caused little effect on the growth of the wild strains. None of the wild and mutant strains were affected by Monceren. On the other hand, fungicides Mertec and Rovral had the highest inhibitory effect. The mutant strains obtained from wild strains Th 11, Th 12 and Tvir showed higher EAC₅₀ and MIC values, being less sensitive to the fungicides evaluated than their respective wild type.

**Key words:** Monceren 250 SF, Mertec 40 SC, Hymexazol 70 WP

**Introduction**

*Trichoderma* fungi, besides being good plant pathogen antagonists, may possess innate resistance to most agrochemicals, including fungicides. However, the level of resistance varies among strains. Some of them have been selected or modified to be resistant to specific agrochemicals (Bolaños, 2004).

Considering the current trend and the increased care for the environment and food safety, it is important to look for effective alternatives, different or complementary to fungicides, where the latter have less or null negative effects when used together with wild or improved bioantagonists, to control plant diseases. The objective of this research was to determine the *in vitro* degree of sensitivity to fungicides used or with potential use to control root and crown rot diseases on tomato, with wild and mutant strains of *Trichoderma*, which have been effective by themselves against *Fusarium oxysporum* f.sp. *lycopersici*, *F. solani*, *Phytophthora nicotianae*, *Pyrenochaeta lycopersici* and *Rhizoctonia solani*, among others.
Materials and methods

*Trichoderma* species used were *T. harzianum* (Th 650, Th V, Th 291, Th 11, Th 12), *T. viride* (Tvir 32), *T. piluliferum* (Tpi 33), and *T. polysporum* (Tpo 34), and the mutants which had a good antagonistic effect against *R. solani* and *P. nicotianae* (Arias, 2005). Fungicides used in the sensitivity tests were Aliette 80 WP (fosetil aluminum), Enzone (sodium tetrathiocarbamate), Hymexazol 70WP (hymexazol), Mertec 40 SC (thiabendazol), Metalaxil 25 DP (metalaxil), Metalaxil MZ 58 WP (metalaxil/mancozeb), Monceren 250 FS (pencycuron), Phyto-fos (neutralized fosforous acid), Rovcap 50% (iprodione+captan), and Rovral 4 FLO (iprodione).

**Mycelium sensitivity**

Serial dilutions of fungicides were added to autoclaved Potato dextrose agar (PDA) (Fenn & Coffey, 1984; Henriquez & Montealegre, 1992), to reach different final concentrations. Once the growing medium was solid, a 1 cm diameter of PDA with the bioantagonist (4-days old) was placed on each Petri dish (Henriquez, 1990). The radial growth of mycelia was measured in cm. A liquid growing medium was prepared for Enzone, sowing immediately and sealing the dishes with parafilm. Temperature and incubation time were 22ºC and 72 hours, respectively.

**Conidial sensitivity**

Effective Average Concentrations (EAC₅₀) were determined for conidia, placing 0.1 mL of 100,000 conidia/mL suspensions on Petri dishes with agar-glucose with growing dosages of the fungicides (Leroux & Gredt, 1981), and incubated 24 hours at 22ºC. Later, 100 conidia were counted under the microscope, where percent germination was determined. Germinated conidia included those with a germination tube of at least five or more times its diameter. For Enzone, the same growing medium and sealing procedure were used, as well as the mycelium sensitivity test.

**Experimental design and statistical analysis**

A completely random experiment design was used in both tests, using four replicates for treatment, and one Petri dish as sample unit for the corresponding treatment. For the *in vitro* sensitivity determination of Effective Average Concentrations (EAC₅₀), Probit analysis was used, and the Minimal Inhibiting Concentration (MIC) was done through polynomial curves.

Results and discussion

None of the wild strains was affected by Monceren 250 FS, including those concentrations higher than the commercial one (Table 1); then, this fungicide could be used in an integrated control program using it together with some of the species and/or mutant strains of *Trichoderma* evaluated.

The ThV wild strain presented the highest EAC₅₀ values with fungicides Metalaxil MZ 58 WP, Enzone, and Phyto-fos, determined by conidia sensibility, demonstrating a high level of tolerance as compared to the other strains evaluated. Strain Th11 showed high levels of tolerance for fungicides Hymexazol and Rovral 4 Flo, with EAC₅₀ values of 509.6 and 522.6, respectively (Table 1). The fungicide effect of Rovral was due to its action on the mycelium (Table 1), when comparing the EAC₅₀ on conidia and the MIC for mycelia of Th11 and Tvir. Fungicide Hymexazol 70 WP was fairly innocuous for both wild and mutant strains of *Trichoderma*, (Tables 1 and 3), considering that the commercial dosage recommended for tomato is 350 ppm a.i. (AFIPA, 2002), as the MIC was not determined due to the low inhibition caused by this fungicide on mycelia of Th11 and Th12. An extremely high sensitivity was observed with Mertec 40 SC on both wild and mutant strains.
Table 1. Wild strains most tolerant to the fungicides evaluated, according to their EAC$_{50}$ on conidia and MIC on mycelium

<table>
<thead>
<tr>
<th>Fungicides</th>
<th>Strains</th>
<th>EAC$_{50}$ (ppm a.i.)</th>
<th>Strains</th>
<th>MIC (ppm a.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monceren 250 SF</td>
<td>n/i</td>
<td>n/i</td>
<td>n/i</td>
<td>n/i</td>
</tr>
<tr>
<td>Metalaxil MZ 58 WP</td>
<td>ThV</td>
<td>4.9</td>
<td>Tpi 33</td>
<td>3005.7</td>
</tr>
<tr>
<td>Aliette 80 WP</td>
<td>Tpo34</td>
<td>200.5</td>
<td>ThV</td>
<td>3183.0</td>
</tr>
<tr>
<td>Rovcap 50%</td>
<td>Tpi 33</td>
<td>9.7</td>
<td>Th 11</td>
<td>217.1</td>
</tr>
<tr>
<td>Hymexazol 70 WP</td>
<td>Th 11, Th 12</td>
<td>509.6</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Mertec 40 SC</td>
<td>Th 650</td>
<td>2.9</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Enzone</td>
<td>ThV</td>
<td>142.2</td>
<td>Tvir 32</td>
<td>1730.7</td>
</tr>
<tr>
<td>Phyto-Fos</td>
<td>ThV</td>
<td>83.8</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Rovral 4 Flo</td>
<td>Th 11, Tvir 32</td>
<td>522.4</td>
<td>Th 11</td>
<td>5.8</td>
</tr>
</tbody>
</table>

n/d: Not determined  n/i: No inhibition

A lower sensitivity of the mutants evaluated was clear for the correspondent wild strains Th11, Th12, and Tvir (Tables 2 and 3). That phenomenon was specifically marked with the mutant strain Th11 A80.1 on Alliete and Mertec in comparison with the wild strain. Also, this mutant strain has demonstrated in antagonistic studies to be very efficacious to control *R. solani* in tomato (Arias, 2005); strain Th12A10.1, which is also efficient for biocontrol of *P. nicotianae* and *R. solani* (Arias, 2005), increased its tolerance to Phyto-fos; and Tvir NG3 decreased its sensitivity to Rovcap (Table 2). This would allow combining strains to handle tomato root diseases in combination with applications of fungicides.

When comparing the level of tolerance (EAC$_{50}$) of the mutant strains with those of their corresponding wild strains (Table 2), for fungicides Aliette 80 WP, Rovcap 50%, Mertec 40 SC, and Phyto-Fos the level of tolerance was increased from 1.3 times with Rovcap for strain Th12 with its mutant Th12A10.1. For Aliette the increase in tolerance in strain Th11 with its mutant Th11 A80.1 was 13.8 times, and for Mertec with the same strain 1.6 times.

Table 2. EAC$_{50}$ for conidia of wild strains and their respective most tolerant mutant strain

<table>
<thead>
<tr>
<th>Fungicides</th>
<th>Wild strains</th>
<th>Mutant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strains</td>
<td>EAC$_{50}$ (ppm a.i.)</td>
</tr>
<tr>
<td>Metalaxil MZ 58 WP</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Alliete 80 WP</td>
<td>Th 11</td>
<td>75.8</td>
</tr>
<tr>
<td>Rovcap 50%</td>
<td>Tvir</td>
<td>6.6</td>
</tr>
<tr>
<td>Rovral 50 WP</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hymexazol 70 WP</td>
<td>Th 11</td>
<td>509.6</td>
</tr>
<tr>
<td>Mertec 40 SC</td>
<td>Th 11</td>
<td>1.1</td>
</tr>
<tr>
<td>Enzone</td>
<td>Th 12</td>
<td>97.1</td>
</tr>
<tr>
<td>Phyto-Fos</td>
<td>Th 12</td>
<td>24.6</td>
</tr>
<tr>
<td>Monceren 250 SF</td>
<td>n/i</td>
<td>n/i</td>
</tr>
<tr>
<td>Rovral 4 Flo</td>
<td>Th 11</td>
<td>522.4</td>
</tr>
</tbody>
</table>

n/d: Not determined  n/i: No inhibition
Fungicides Hymexazol 70 WP, Enzone, and Rovral 4 Flo, when comparing their EAC50, there was a decrease in tolerance of the mutant strains with respect to their wild strains, (1.3 times for Hymexazol, 2.0 times for Enzone, and 26.1 times for Rovral 4 Flo).

Table 3. MIC for mycelia of wild strains and their most tolerant mutant strains

<table>
<thead>
<tr>
<th>Fungicides</th>
<th>Wild strains MIC (ppm a.i.)</th>
<th>Mutant strains MIC (ppm a.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metalaxil MZ 58 WP</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Aliette 80 WP</td>
<td>Tvir 3083.1</td>
<td>Tvir NG19 4179.7</td>
</tr>
<tr>
<td>Rovcap 50%</td>
<td>Th 11 217.1</td>
<td>Th 11A20.1 172.0</td>
</tr>
<tr>
<td>Rovral 50 WP</td>
<td>Th 11A80.1 35.1</td>
<td>Th 11A160.1 7010.9</td>
</tr>
<tr>
<td>Hymexazol 70 WP</td>
<td>Th 11 n/d</td>
<td>Th 11A20.1 5899.3</td>
</tr>
<tr>
<td>Mertec 40 SC</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Enzone</td>
<td>Th 11 n/d</td>
<td>Th 11A20.1 5899.3</td>
</tr>
<tr>
<td>Phyto-Fos</td>
<td>s/i</td>
<td>n/i</td>
</tr>
<tr>
<td>Monceren 250 SF</td>
<td>s/i</td>
<td>n/i</td>
</tr>
<tr>
<td>Rovral 4 Flo</td>
<td>Th 12 n/d</td>
<td>Th 12A10.1 30.9</td>
</tr>
</tbody>
</table>

n/d: Not determined  n/i: No inhibition

Acknowledgements

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References


Improving control of storage diseases on apple by combining biological and physical post-harvest methods

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² Bionext, Passage des déportés, 2, 5030 Gembloux, Belgium, e-mail: pps@bionext.be;
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Abstract: Post-harvest non-chemical treatments consist of a large range of different approaches, including strengthening of the commodity’s natural defence mechanisms, thermotherapy, application of antagonistic microorganisms and natural antimicrobial substances. NEX0101 is a promising antagonistic biocontrol agent containing the yeast *Candida oleophila* as the active ingredient. NEX0101 was developed by Bionext, a spin-off from the laboratory of Prof. H. Jijakli, and is currently evaluated for commercial use. The product contains a yeast strain isolated from apple fruit and was originally developed for the control of post-harvest diseases on apple (Jijakli et al., 2003). The antifungal effectiveness of this antagonist can be increased by addition of calcium salts (Van Laer et al., 2004, Jijakli et al., 2006). As the mode of action of this yeast is based on the colonisation of wounds, the mean targets of NEX0101 are blue mould, caused by *Penicillium expansum*, and grey mould, caused by *Botrytis cinerea*. 

Facing possible latent infections, thermotherapy by using hot water treatments could provide an advanced control towards lenticel rot (*Pezicula* spp., anamorph *Gloeosporium* spp.). A combination of both physical and biological treatment techniques could broaden the spectrum to all key pathogens on apple and pear. According to previous results the combination NEX0101 with calcium gluconate provides an advanced mould control towards *P. expansum*. All treated objects gave a significant reduction in decay. A submersion time of 2 minutes proved to be slightly more efficient compared to 30 seconds dipping. Facing the treatments techniques, no clear difference was observed comparing dipping or drenching the apple fruits. The best results were achieved using NEX0101 in combination with post-harvest dipping by thermotherapy. The hot water treatment alone was statistically inefficient towards wound parasite *P. expansum*, on the contrary thermotherapy stimulates the decay caused by this post-harvest pathogen. For the future a combination of biological and physical treatments could offer a worthy non-chemical alternative for organic and integrated fruit growers towards fruit rot decay, although more research is necessary to implement these methods in practice.

Key words: biological control, *Candida oleophila*, *Penicillium expansum*, post-harvest decay

Introduction

The purpose of this trial was to compare biological and physical post-harvest methods of storage disease control on apples, and potentially to improve it by combining them. Concerning the biological control strategy, NEX0101 is included in the trial setup. This promising antagonistic biocontrol agent contains the yeast *Candida oleophila* strain O as the active ingredient and is currently evaluated for commercial use. The antifungal effectiveness of this antagonist can be increased by addition of specific additives. As the mode of action of
this yeast is based on the colonisation of wounds, the mean targets of NEX0101 are blue mould, caused by *Penicillium expansum*, and grey mould, caused by *Botrytis cinerea*.

As for the physical control strategy, thermotherapy using hot water treatment was included in this trial. Facing potent latent infections, thermotherapy could provide an advanced control towards lenticel rot (*Gloeosporium* spp.). A combination of both physical and biological treatment techniques could broaden the spectrum to all key pathogens on apple and pear.

**Material and methods**

The trial was executed on apple variety Golden delicious. The fruits originated from one orchard and were stored till the start of the trial, under standard storage conditions (1°C) at the location Gorsem of Proefcentrum Fruitteelt. The fruits were sorted on fruit size and injured apples were removed. Apples with a fruit size of 75-85 mm were intended to be used in the trial.

**Artificial wounding**

The fruits were infected with the storage disease *P. expansum*. This fungus is a wound parasite, for infection the fruits need to be wounded in advance. In concrete the apples were punctured at 4 places, divided equally over the fruit, using a sterilized nail, mounted in a cork plug, creating a 1-mm wide by 2-mm deep cylindrical wound. As the fruits were dipped before the artificial infection, the trial was set up to evaluate the preventive efficiency of the test objects.

**Post-harvest dipping/drenching treatment**

The wounded fruits were dipped in the treatment solution containing the test product NEX0101. To prepare the test solution the biomass granules of NEX0101 (WG formulation containing $3 \times 10^{10}$ CFU/g) have been reactivated in a small volume of water (13.33 g in 0.5 l). The suspension was smoothly agitated for one hour to homogenize. As the antifungal effectiveness of this biocontrol agent can be increased by addition of the specific additive calcium gluconate, the trial was set up using the biomass in combination with this additive, at a dose rate of 2 g/l. NEX0101 was used at the target concentration of the biomass of 0.033% = $1 \times 10^7$ CFU/ml. Each test object contains 4 replicates of 50 fruits. Next to the untreated dry check, one water dipped check object was included and dipped for 2 minutes, just as was done for NEX0101. Also one object was included facing a dipping time of 30 seconds. Dipping was done in plastic baskets with a content of 60 l and filled up with 40 l of the test solution. Perforated baskets containing 50 fruits were dipped in the test solution. In the trial setup also one object was included using post-harvest drenching. For this, spray equipment with a high volume was used. The construction consists of a spray tower and a moving table. In the spray tower, 3 different nozzles are available, one above the fruits (spray tip: Twin Jet TJ-60 8004 VS) and two sideways (spray tip: Teejet UB8502 SS) guaranteeing a high flow rate (drenching). The fruits were placed on the moving table (0.045 m/s) and treated with the test suspension (spray pressure 3 bar). Next to the biological control of *P. expansum* also a physical control strategy was evaluated. In the trial setup, two objects were included facing thermotherapy (a hot water treatment for 2 min. at 50°C) respectively alone or in combination with a biological dip treatment with NEX0101. An incubation time of about one hour was respected between both post-harvest dip treatments. After all objects were treated, the fruits were placed in small wooden boxes (50 fruits/box = 1 replicate), marked and wrapped in plastic foil leaving holes to insure a high level of humidity. The boxes were placed at random on pallets in a 100% humidified room (with a mist defensor) and incubated for 24 h at about 20°C.
Artificial infection

After this 24 hours incubation, the fruits were inoculated with a conidial suspension of *P. expansum*. The conidial suspension was made from a culture plated on PDA-agar (Potato dextrose agar 39 g/l, Merck, Germany). The conidia of 10 days old *Penicillium* cultures, sporulated under normal light, were used to inoculate the fruits. The conidiating plates were rinsed using sterilized demineralised water, containing 0.05% of Tween 20 (Fluka Chemie, Switzerland). After counting in a Burker cell, the conidial suspension was assessed, by controlling the germination of the conidia on a PDA-agar. In this trial the germination level was more than 90%. To infect the fruits, spray equipment was used. The construction (spray tower and a moving table) was also used for the object using post-harvest drenching. Only some preset parameters have been adjusted: in the spray tower 3 flat spray tips (TeeJet 650050, spraying angle 65°) are installed and the speed of the mobile table amounts to 0.19 m/s. The fruits were placed on the moving table and infected with the conidial suspension (spray pressure: 2 bar). When they were passed the nozzles, the fruits were turned and infected in the same way on the other side. Afterwards the fruits were collected again in wooden boxes and wrapped in plastic foil leaving holes to insure a high level of humidity. The boxes were stored on pallets in a 100% humidified room (with a mist defensor) and incubated for 48 hours at room temperature (20°C). The wooden boxes were arranged at random on the pallets. On day four, the pallets were stored in a cold storage room at 1°C for one week. Afterwards the pallets were placed at 20°C and regularly moistened till wet to accelerate the development of the disease.

Assessment on fruit rot decay

About 18 days later the degree of decay of the apple fruits was evaluated. A second assessment of *Penicillium* took place one week later. To assess the wounded fruits on decay, per fruit the diameter of the lesion area is measured for each of the 4 wounds (mm). The total diameter of the lesions is expressed per fruit and when the whole fruit was infected with the disease, a total maximum diameter of 200 mm was noted. The efficacy (Abbott value) of the test substances is calculated on the total diameter of lesions on 50 fruits. The number of infected fruits and the percentage of infected fruits were also calculated.

Results and discussion

A successful infection with *P. expansum* occurred with 43.5% of infected apple fruits in the untreated dry check. The total diameter of all infected fruits amounted here to 1393.8 mm on a total of 50 fruits. NEX0101 provided an advanced mould control towards *P. expansum*. All NEX0101 treated objects gave a significant reduction in decay. All objects containing NEX0101 gave efficacies with no significant differences, whatever the dipping time (2’ or 30’’), the application technique (dipping or drenching) or the combination with thermotherapy. The hot water treatment alone was clearly inefficient towards wound parasite *P. expansum*, on the contrary thermotherapy stimulates the decay caused by this post-harvest pathogen. In this object 63.0% of all fruits were infected compared to 43.5% in the non-treated dry check. Hot water treatments has no persistent activity on infections later on. The best results were achieved using NEX0101 in combination with post-harvest dipping by thermotherapy (89.4% efficacy). Also in an experiment on grapefruit, the combination of a hot water treatment with a biological agent dramatically reduced the decay by *P. digitatum* compared with each treatment alone (Droby, 2006).

For the future a combination of biological and physical treatments could offer a worthy consumerfriendly non-chemical alternative for fruit growers towards fruit rot decay. Such
strategies are needed to reduce the dependence on fungicides in the organic and integrated fruit production (Creemers et al., 2006).

Table 1. Evaluation of *Penicillium* fruit rot infestation on the apple cultivar Golden delicious

<table>
<thead>
<tr>
<th>Object</th>
<th>Dose (%)</th>
<th><em>P. expansum</em> Infection (%)</th>
<th>Diameter total lesions</th>
<th>Efficacy ABB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Check dry</td>
<td></td>
<td>43.5 bc</td>
<td>1393.8 b</td>
<td></td>
</tr>
<tr>
<td>2 Check water treated</td>
<td></td>
<td>29.5 b</td>
<td>891.3 b</td>
<td></td>
</tr>
<tr>
<td>3 NEX0101 dipping 2’</td>
<td>0.033</td>
<td>8.5 a</td>
<td>201.3 a</td>
<td>85.6</td>
</tr>
<tr>
<td>Biomass + Additive</td>
<td>0.200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 NEX0101 dipping 30’’</td>
<td>0.033</td>
<td>12.5 a</td>
<td>312.5 a</td>
<td>77.6</td>
</tr>
<tr>
<td>Biomass + Additive</td>
<td>0.200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 NEX0101 drenching</td>
<td>0.033</td>
<td>9.0 a</td>
<td>213.8 a</td>
<td>84.7</td>
</tr>
<tr>
<td>Biomass + Additive</td>
<td>0.200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Thermotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2’ in hot water (50°C)</td>
<td>63.0 c</td>
<td></td>
<td>1923.8 c</td>
<td>-38.0</td>
</tr>
<tr>
<td>7 Thermotherapy 2’ + dipping NEX0101 (2’)</td>
<td>0.033</td>
<td>7.5 a</td>
<td>147.5 a</td>
<td>89.4</td>
</tr>
<tr>
<td>(Biomass+Additive)</td>
<td>0.200</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical analyses were performed using the Unistat Statistical Package, version 5.5 (Unistat Ltd. 1998, London, England).

**Acknowledgement**

Research centre subsidized by the Ministry of the Flemish Community.

**References**


Management of cucurbit powdery mildew on greenhouse-grown melons by different biological control strategies

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Abstract: Podosphaera fusca is the causal agent of cucurbit powdery mildew in Spain. Control failures of many commercial fungicides have favoured the progress of biological control approaches and their integration in feasible integrated pest management programs. In this study, the ability of two mycoparasites and four strains of Bacillus subtilis alone or in alternation with a fungicide (azoxystrobin) to efficiently manage P. fusca under greenhouse conditions is provided, supporting their use as parts of integrated control programmes for management of cucurbit powdery mildew.

Key words: Bacillus subtilis, mycoparasites, Podosphaera fusca

Introduction

Powdery mildew is a common disease of cucurbits under field and greenhouse conditions in most areas of the world, causing serious economical losses. The disease is clearly diagnosed by white, powdery mold development on both leaf surfaces, petioles and stems (Jarvis et al., 2002). Podosphaera fusca and Golovinomyces cichoracearum are the two most commonly recorded fungal species causing cucurbit powdery mildew; however, to date P. fusca has been identified as the sole cause of the disease in Spain (Del Pino et al., 2002). Control failures of many commercial fungicides due to the development of pathogen resistance have favoured the progress of biological-control approaches to manage the disease (Kiss, 2003; Fernández-Ortuño et al., 2006). Biological agent performance depends strongly of high relative humidity and often is lower compared with efficacy of chemicals, so their implementation in cucurbit powdery mildew management requires exploring appropriate tactics which may combine biocontrol agents with different mode of actions and chemicals (Paulitz & Bélanger, 2001). For biocontrol of powdery mildews, mycoparasites have been, so far, the most explored approaches (Kiss, 2003), and although less studied, the use of antibiotic-producing microorganisms have been reported to provide an adequate control of cucurbit powdery mildew (Paulitz & Bélanger, 2001). We have previously reported the ability of two mycoparasitic fungi, Ampelomyces quisqualis (AQ10®, Ecogen) and Lecanicillium lecanii (Mycotal®, Koppert Biological Systems) and four strains of Bacillus subtilis to manage efficiently powdery mildew under different relative humidity conditions in seedling trials conducted in growth chambers (Romero et al., 2004a). The aim of this study was to determine the efficacy of those biocontrol agents against cucurbit powdery mildew on melon plants grown under greenhouse conditions.
Material and methods

Pathogen and biocontrol agents
Isolate SF26 of *P. fusca* race 1 was routinely grown *in planta* on cotyledons of zucchini cv. Negro Belleza and maintained *in vitro* as described elsewhere (Romero et al., 2004b). The mycoparasitic fungi *A. quisqualis* and *L. lecanii* were used as the formulated products AQ10® (Ecogen, Langhorne, PA, USA) and Mycotal® (Koppert Biological Systems, Berkel, The Netherlands), respectively. The *B. subtilis* strains, UMAF6614, UMAF6639 and UMAF8561, were routinely grown in nutrient agar from frozen stocks and batch fermented in a 5L bioreactor (Oh et al., 1995; Romero et al., 2004b).

Greenhouse assays
Three biocontrol experiments were conducted in an experimental greenhouse during the spring and autumn of 2005 and spring of 2006. The different treatments were arranged in a completely randomised block design, providing at least three replicates of four plants for each treatment. The following treatments were tested: i) control untreated plants; ii) plants treated with tap water; iii) Ortiva (azoxystrobin); iv) AQ10 in 0.25% ADDIT (mineral oil); v) Mycotal in ADDIT; vi) 0.25% ADDIT; vii) *B. subtilis* UMAF6614; viii) *B. subtilis* UMAF6639; ix) *B. subtilis* UMAF8561, x) alternating Ortiva as the first application and *B. subtilis* UMAF6614 as the second, xi) AQ10 and xii) Mycotal without oil. Three to four leaves per plant were inoculated with *P. fusca* by spraying a conidia suspension (10⁴ conidia/ml). First application of the treatments was done when the first mildew colonies were observed, and the second application ten days later. Spore suspension of mycoparasites was prepared at 5 10⁶ spores/ml as previously described (Romero et al., 2003) and bacterial suspensions were adjusted to 10⁷-10⁸ cfu/ml (Romero et al., 2004b).

Disease assessment and data analysis
Disease severity estimated as the percentage quantification of the leaf area covered by powdery mildew, and degree of powdery mildew sporulation deduced from conidia counts at the end of each greenhouse experiment were assessed as reported earlier (Romero et al., 2003, 2004b).

Results and discussion
Inoculation of plants with *P. fusca* conidia suspensions adjusted to 10⁴ conidia/mL provoked disease severity index ranging from 60 to 80% of leaf area covered by powdery mildew after 30 days (Table 1) which linked profuse sporulation (1200-1800 conidia cm⁻²) as shown in Table 2.

In general all the biological treatments reduced strongly disease severity compared to the untreated controls (Table 1), but the best results were achieved in experiment II conducted in autumn 2005, when it was registered the lower oscillations in relative humidity. Similarly, conidia production was practically abolished by all biological treatments (Table 2), obtaining sporulation reduction indexes around 90%, which highlight the relevance to reduce pathogen sporulation in order to efficiently manage the disease (Paulitz & Bélanger, 2001). As expected, mycoparasite effectiveness turned out to be absolutely dependent on mineral oil ADDIT, showing disease reductions of 80-95%. In absence of mineral oil, however, severity values obtained by mycoparasites were not statistically different from untreated or water controls (data not shown). The fact that mycoparasites efficiently control powdery mildew in combination with oil suggested a combinatorial action between both treatments and supported the previously reported double role for oils, retaining high relative humidity values on the leaf optimal for mycoparasites and inducing damage to *P. fusca* conidia (Dik et al., 1998). The
bacterial suspensions mainly composed by vegetative cells were also very effective on disease control, achieving disease reductions similar to mycoparasites with mineral oil, suggesting vegetative cells of *B. subtilis* rather than endospores are responsible for disease protection ability (Collins & Jacobsen, 2003).

Table 1. Suppressive effect of different biological control treatments on powdery mildew severity in greenhouse-grown melon plants. Disease severity expressed as percentage of leaf area covered by powdery mildew was recorded 30 days after pathogen challenge. Percentage of disease reduction achieved by treatments referred to untreated controls is also indicated.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Experiment I Severity Reduction</th>
<th>Experiment II Severity Reduction</th>
<th>Experiment III Severity Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>65 a x</td>
<td>78 a</td>
<td>81 a</td>
</tr>
<tr>
<td>Water</td>
<td>63 a</td>
<td>77 a</td>
<td>80 a</td>
</tr>
<tr>
<td>Ortiva</td>
<td>4 d</td>
<td>2 b</td>
<td>2 b</td>
</tr>
<tr>
<td>AQ10 + ADDIT</td>
<td>9 bcd</td>
<td>4 b</td>
<td>18 c</td>
</tr>
<tr>
<td>Mycotal + ADDIT</td>
<td>6 cd</td>
<td>2 b</td>
<td>12 bc</td>
</tr>
<tr>
<td>ADDIT</td>
<td>11 bc</td>
<td>3 b</td>
<td>34 d</td>
</tr>
<tr>
<td><em>B. subtilis</em> UMAF6614</td>
<td>7 bcd</td>
<td>2 b</td>
<td>8 b</td>
</tr>
<tr>
<td><em>B. subtilis</em> UMAF6639</td>
<td>10 bcd</td>
<td>2 b</td>
<td>11 b</td>
</tr>
<tr>
<td><em>B. subtilis</em> UMAF8561</td>
<td>12 b</td>
<td>3 b</td>
<td>9 b</td>
</tr>
<tr>
<td>1st Ortiva, 2nd UMAF6614</td>
<td>–</td>
<td>2 b</td>
<td>2 b</td>
</tr>
</tbody>
</table>

Note: Mean values followed by different letters within a column were significantly different accordingly to the LSD test \((P=0.05)\).

Table 2. Effect of different biological control treatments on *P. fusca* conidiation in greenhouse-grown melon plants. Conidiation degree was recorded 30 days after pathogen inoculation and expressed as conidia cm\(^{-2}\). Percentage of sporulation reduction achieved by treatments referred to untreated controls is also indicated.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Experiment I Conidia Reduction</th>
<th>Experiment II Conidia Reduction</th>
<th>Experiment III Conidia Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1333 a x</td>
<td>1595 a</td>
<td>1812 a</td>
</tr>
<tr>
<td>Water</td>
<td>1285 a</td>
<td>1173 a</td>
<td>1778 a</td>
</tr>
<tr>
<td>Ortiva</td>
<td>81 b</td>
<td>66 b</td>
<td>83 c</td>
</tr>
<tr>
<td>AQ10 + ADDIT</td>
<td>138 b</td>
<td>80 b</td>
<td>388 bc</td>
</tr>
<tr>
<td>Mycotal + ADDIT</td>
<td>49 b</td>
<td>32 b</td>
<td>272 bc</td>
</tr>
<tr>
<td>ADDIT</td>
<td>170 b</td>
<td>53 b</td>
<td>634 d</td>
</tr>
<tr>
<td><em>B. subtilis</em> UMAF6614</td>
<td>123 b</td>
<td>56 b</td>
<td>133 c</td>
</tr>
<tr>
<td><em>B. subtilis</em> UMAF6639</td>
<td>192 b</td>
<td>132 b</td>
<td>192 c</td>
</tr>
<tr>
<td><em>B. subtilis</em> UMAF8561</td>
<td>137 b</td>
<td>90 b</td>
<td>313 bc</td>
</tr>
<tr>
<td>1st Ortiva, 2nd UMAF6614</td>
<td>–</td>
<td>81 b</td>
<td>116 c</td>
</tr>
</tbody>
</table>

Note: Mean values followed by different letters within a column were significantly different accordingly to the LSD test \((P=0.05)\).
Several *B. subtilis* strains are receiving great attention because of their versatility to exert protection to plants. Early studies pointed out antibiosis could be involved in the disease protection by these *B. subtilis* strains (Romero et al., 2004). Combination of biologicals with other control strategies has aroused the attention of plant pathologists as an interesting way to overcome the main constraints related to biocontrol agents. In our study, alternation of fungicide Ortiva with *B. subtilis* UMAF6614 provided disease protection statistically comparable to chemical control (Ortiva).

Our results clearly prove that powdery mildew in greenhouses cucurbits can be properly managed by the use of biological means. Furthermore, *B. subtilis* strains could rationally be combined with fungicides. Further research on the biocontrol effectiveness of these microorganisms under field and greenhouse conditions and their interactions with other strategies of disease control prior to developing suitable integrated control strategies focused to minimize the dependence on chemicals of cucurbit powdery mildew management are being currently undertaken.

**Acknowledgements**

This study was supported by grants from Plan Nacional de Recursos y Tecnologías Agroalimentarias from Ministerio de Educación y Ciencia, Spain (AGL2001-1837 and AGL2004-06056). D. Romero was supported by a grant from the former Ministerio de Ciencia y Tecnología.

**References**


Effect of application time of control agents on *Podosphaera aphanis* and side effect of fungicides on biocontrol agents survival on strawberry leaves

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\(^2\)Department of Plant Pathology and Weed Research, ARO, The Volcani Center, Bet Dagan 50250, Israel

**Abstract:** Powdery mildew of strawberry (*Podosphaera aphanis* f.sp. *fragariae*) is one of the main concerns worldwide either in all strawberry growing systems. Integrated pest management allows adequate disease control while reducing the use of chemical fungicides. The optimal integration between chemical and biocontrol agents (BCAs) is based on the right timing of treatments according their mechanism of action and the disease stage and level and, since BCAs are living organisms, on avoiding negative side effect of chemicals on them. With the final aim of integrating chemicals and BCAs in a decision support system for optimization of IPM on strawberry the efficacy of some widely used fungicides (sulphur, polyoxin, kresoxim methyl and tetraconazole) and some Application of BCAs (*Ampelomyces quisqualis* AQ10, *Bacillus subtilis* QST 713, *Trichoderma harzianum* T39) against powdery mildew at different times in respect to pathogen infection was evaluated. Sulphur and *B. subtilis* QST 713 were active only if applied at inoculation time; polyoxin, kresoxim methyl and tetraconazole had the highest efficacy if applied after inoculation. *T. harzianum* T39 was active when applied 4 days before inoculation and it was not active when applied after inoculation suggesting a mechanism of action involving resistance induction in the host plant. *A. quisqualis* showed a poor efficacy probably due to the high disease pressure and, being a hyperparasite, to early application vs. pathogen establishment. Side effects of chemicals fungicides on BCAs were also evaluated. Quantitative survival of *T. harzianum* T39 and *B. subtilis* QST 713 on strawberry leaves was not affected by fungicides (sulphur, penconazole, azoxystrobin, polyoxin, kresoxim methyl and tetraconazole) treatment, while *A. quisqualis* viability decreases significantly.

**Key words:** powdery mildew

**Introduction**

Strawberry powdery mildew (SPM) is caused by the obligate pathogenic fungus *Podosphaera aphanis* f. sp. *fragariae*. It causes severe losses world-wide in traditional cropping systems and in greenhouse soil-free systems. It control is traditionally based on chemical fungicides in the Mediterranean area.

Biocontrol agents (BCAs) may be good alternatives to chemical fungicides if they offer acceptable levels of disease control. Integrated pest control strategies using BCAs alternated, followed, or preceded by chemical fungicides could reach a sufficient disease control while reducing the use of chemical fungicides. Optimal integration of BCAs with chemical requires the right timing of treatments in order to avoid side effects of the chemical on the BCA. In particular *Trichoderma harzianum* T39 (Elad, 2000) and *Ampelomyces quisqualis* (Kiss et al., 2004; Sztejnberg et al., 1989) both fungi, could possibly be affected by chemical fungicides.
We evaluated the efficacy of powdery mildew suppression by some widely used chemical fungicides and some BCAs applied at different times in relation to the inoculation time by the pathogen. We tested the quantitative survival of *T. harzianum* T39, *A. quisqualis* and *Bacillus subtilis* on strawberry leaves treated prior with chemical fungicides. The final objective of this work was to produce data allowing the integration of chemicals and BCAs in a decision support system for the optimization of IPM on strawberry.

**Material and methods**

*Application time of control agents on Podosphaera aphanis*

Efficacy of control agents (CAs) under greenhouse conditions was evaluated. Fungicides (tetraconazole, polyoxin, kresoxim methyl, sulphur) and BCAs (*A. quisqualis* AQ10, *T. harzianum* T39, *B. subtilis* QST 713) were tested. Trials were performed on potted strawberry plants cv. Elsanta under controlled greenhouse conditions (25±5°C and 60±10% RH). Test products, references and untreated control were arranged in fully randomised blocks, five replicates of one plant for each treatment. Fungicides and BCAs were applied at different times (4, 0, 3, 7 and 14 days) in relation to the inoculation (time 0). CAs used were (Heliosoufre 3 ml/l, Polar 0.5 g/l, Domark combi 5.0 g/l, Stroby 1.0 g/l, AQ10 0.08 g/l, Trichodex 10 g/l, Serenade 4.0 g/l). Inoculation was done by shaking leaves collected in the field presenting heavy sporulating mildew lesions above the test plants. Assessments were carried out 14 and 21 days after inoculation. Five randomly chosen leaves per replicate were scored. Disease severity of *P. aphanis* (percentage diseased leaf area) and incidence (percentage of leaves with disease symptoms) were scored. Analysis of variance on arcsin transformed data (ANOVA) was used. Significant differences among treatments were determined by Fisher’s test at *P*<0.05.

*Survival of T. harzianum T39, B. subtilis and A. quisqualis on leaf surface after chemical treatment*

The side effect of fungicides (sulphur, penconazole, azoxystrobin, polyoxin, Kresoxim methyl and tetraconazole) on *T. harzianum* T39, *B. subtilis* QST 713 and *A. quisqualis* were tested on strawberry leaves by determining their survival rate in presence of the fungicides. Cell suspension (1×10^6/ml) of each microorganism was sprayed on leaves 3 hours before chemical fungicides sprays. Plants were arranged in fully randomised blocks, five replicate plants per treatment. Leaf disks were cut at different times (3, 4, 72 and 192 hours after treatments) and microorganisms were recuperated by washing with sterile water. These suspensions were diluted, plated on semi-selective media and incubated at 25-27°C for 48 hours. *T. harzianum* T39 and *B. subtilis* QST 713 CFU/ml were counted to assess microorganisms’ survival. For *A. quisqualis*, leaves were detached at the above mentioned times, incubated at 26°C for 48 hours, pealed with cello-tape and conidial germination was assess under light microscope. Analysis of variance (ANOVA) was used to analyze the results. Significant differences among treatments were determined by Tukey’s test.

**Results and discussion**

*Effect of application time of CAs on P. aphanis*

Kresoxim methyl, tetraconazole and polyoxin reduced the disease if applied after pathogen inoculation (Table 1). Similar result was seen for other chemical pesticides (penconazole and azoxystrobin) belonging to the same chemical group of tetraconazole and kresoxim methyl, respectively (Fiamingo et al., 2005). Sulphur is a good preventive product, but not adequately effective when plants are treated after infections. *T. harzianum* T39, probably acting as
resistance inducer, controls better the disease if applied before its establishments; B. subtilis QST 713, which seems to produces active metabolites against pathogens, reduces SPM if applied at inoculation time, while A. quisqualis, being and hyperparasite, needs the presence of the pathogen in order to be established and active.

Table 1. Severity (%) of strawberry powdery mildew on leaves of plants treated with chemicals and bio control agents applied at different time in relation to the inoculation time

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Treatment time (day related to inoculation (days))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-4</td>
</tr>
<tr>
<td>Water**</td>
<td>41.6 b</td>
</tr>
<tr>
<td>Sulphur</td>
<td>27.5 ab</td>
</tr>
<tr>
<td>Polioxyn</td>
<td>27.8 ab</td>
</tr>
<tr>
<td>Tetraconazole+S</td>
<td>20.3 ab</td>
</tr>
<tr>
<td>Kresoxim methyl</td>
<td>20.3 ab</td>
</tr>
<tr>
<td>Ampelomyces quisqualis</td>
<td>24.4 ab</td>
</tr>
<tr>
<td>Trichoderma harzianum</td>
<td>19.3 a</td>
</tr>
<tr>
<td>Bacillus subtilis QST 713</td>
<td>29.5 ab</td>
</tr>
</tbody>
</table>

Numbers in each column followed by the same letter do not differ with $P \leq 0.05$ (Fisher’s test).

* 2 hours; ** untreated control.

![Figure 1](image1.png)

Figure 1. Survival of Bacillus subtilis QST 713 (left) and Trichoderma harzianum T39 (right) on strawberry leaves surface applied 3 h prior to the application of the chemical fungicide as assessed by the serial dilution plating method and colony forming units. (S2= Heliosoufre).

**Side effects of chemical fungicides on BCAs survival**

Viability expressed as CFU of B. subtilis QST 713 and T. harzianum T39, applied separately on strawberry leaves surface, declined rapidly to low levels after 3 days is described in Fig. 1.

The initial B. subtilis QST 713 population on leaves of untreated control (water) 3 hours after application, was $1 \times 10^7$ CFU/cm², and dropped after 72 hours down to $4.4 \times 10^5$ CFU/cm². Similarly T. harzianum T39, dropped in the untreated control, from initial values of $2.1 \times 10^5$ CFU/cm² (after 3 hours), to $6.3 \times 10^4$ after 72 hours. Furthermore, the decline of B. subtilis QST 713 cell population was even faster than that of T. harzianum, in the first 72 hours.
Survival of *A. quisqualis* decrease also with time (data not shown), and data collected show low conidial germination rate. In the control (water) 6 h after its application the conidial germination rate equal 7.9%.

Chemical fungicides sprayed 3 hours after *B. subtilis* QST 713 and *T. harzianum* application do not affect population survival. Differently, *A. quisqualis* conidial germination rate was significantly reduced by fungicides application, showing its immediate susceptibility to all the different chemical applications (Table 2).

Table 2. Effect of a fungicide application on the germination rate of *Ampelomyces quisqualis* (%) on strawberry leaves. Fungicides were applied 3 h after *A. quisqualis*.

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Time after fungicide treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Water</td>
<td>7.9 a</td>
</tr>
<tr>
<td>Sulphur</td>
<td>0.1 b</td>
</tr>
<tr>
<td>Azoxystrobin</td>
<td>0.4 b</td>
</tr>
<tr>
<td>Penconazole</td>
<td>1.5 b</td>
</tr>
<tr>
<td>Polyoxin AL 50%</td>
<td>1.9 b</td>
</tr>
<tr>
<td>Tetraconazole+Sulphur</td>
<td>0.2 b</td>
</tr>
<tr>
<td>Kresoxim methyl</td>
<td>0.6 b</td>
</tr>
</tbody>
</table>

Numbers in each column followed by a common letter do not differ to each other according to Tukey’s test at $P \leq 0.05$.

**Acknowledgements**

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**References**


Integration of biocontrol agents and natural products against tomato late blight

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Abstract: Late blight (Phytophthora infestans) is one of the most devastating diseases of potato and tomato. It can result in complete destruction of these crops. The aim of this study was to test alternatives to synthetic fungicides currently used in the control of late blight and to evaluate the efficacy of a combined use of biocontrol agents and natural products. Antifungal activities of the BCAs Trichoderma harzianum T39 (formulated), four yeast and five bacteria isolates from Israel and Italy were assayed on tomato and potato plants artificially inoculated with P. infestans sporangia, under growth chamber and greenhouse conditions. Additionally we tested the effect of an alcoholic plant extract (Elot-vis), a chitosan based product (Chitoplant) and a fatty acid based agent (Tecnobiol) compared to two low copper rate products (Labicuper and Labimethyl) and copper hydroxide (Kocide). Significant control of the disease on tomato was observed following treatments with T. harzianum T39, two bacteria isolates (B19 and B69), Chitoplant and Elot-vis. T. harzianum T39, Elot-vis and Chitoplant were consistent in their control activity. The tests in potato consisted only of the BCAs. T39 and B19 significantly reduced potato late blight. In tomato a synergistic effect was obtained for B19 plus Elot-vis.

Key words: efficacy assay, friendly control agents

Introduction

Late blight (Phytophthora infestans) is one of the most devastating diseases of potato and tomato. It can result in complete destruction of these crops. Late blight appears on potato or tomato leaves as pale green, water-soaked spots, sometimes surrounded by a pale yellowish-green border that merges with healthy tissue. Lesions enlarge rapidly and turn dark brown to purplish-black on leaves and stems. In high humidity conditions, a cottony, white mould growth with sporangia is usually visible (Powelson & Inglis, 1998).

In recent years, highly aggressive strains of this disease, many of them insensitive to widely used synthetic fungicide, have created major problems to potato and tomato growers (Powelson & Inglis, 1998). Good agronomic practices and tolerant varieties can be used to reduce the disease pressure. Copper sprays are commonly used against late blight in organic production. Several commercial copper products are available and 9 to 15 sprays are often necessary to control the disease in areas where environmental conditions are favourable for infections. This raises the issue of copper accumulation in the soil where its toxicity for micro and macroorganisms is not compatible with sustainable production. Organic growers are
encouraged to adopt an integrated approach to control late blight, which include alternatives, such as natural product or biocontrol agents (Kuepper & Sullivan, 2004).

Chitosan exhibits elicitor activity and induces both local and systemic resistance (Perekhod et al., 1997) and it can successfully control *P. infestans* in potatoes (Abd-El-Kareem et al., 2005). Elot-vis is a commercial preparation based on plant extract active against *P. infestans* increasing plant defence mechanism and inducing resistance (Stephan et al., 2005). The aim of this study was to test alternatives to synthetic fungicides currently used in the control of late blight and to evaluate their combination. Biocontrol agents (BCAs) were combined with natural resistance inducers that are supposed not to be toxic to living microorganisms.

### Material and methods

Trials were carried on tomato under controlled conditions. Experiments were repeated at least twice. Tomato plants were planted in peat:volcanic gravel mixture in pot and grown in controlled temperature chamber until they showed at least 5 fully developed leaves. Plants were artificially infected by spraying a water suspension of *P. infestans* sporangia that were produced on tomato plant material. For this purpose, symptomatic leaves were collected and kept for 6 days in a humidity chamber. Afterwards 50 ml of water was added to sporulating leaves and kept at 5°C for 1 h. Suspension was diluted to obtain a final concentration of 5×10² conidia/ml. Inoculated plants were immediately enclosed in polyethylene bags for 24 h.

Bacteria and yeasts were respectively grown on nutrient broth (Oxoid) and yeast malt dextrose broth (3 g of malt extract, 3 g of yeast extract, 5 g of peptone, and 10 g of dextrose per litre) liquid medium at 120 rpm for 48 h at 25°C. Afterwards they were centrifuged twice and re-suspended in water obtaining a concentration of 5×10⁷ cells/ml (yeasts) and 1×10⁸ cells/ml (bacteria). A concentration of 4 g/l of *Trichoderma harzianum* T39 (formulated) was used (10⁶ conidia/ml).

Antifungal activities of the BCAs, four yeasts (Y13, Y89, Y16, Y2) and five bacteria (B69, B71, B19, B6, F77) isolated in Israel and Italy were tested. The efficacy of an alcoholic plant extract (Elot-vis; Prophyta, Germany) used at the concentration of 5 ml/l; a chitosan based product (Chitoplant; ChiPro Gmbh Germany) used at 0.5 g/l; and a fatty acid based agent (Tecnobiol; Tecnotrosa, Italy) used at 10 g/l were tested. Two low copper rate products (Labicuper and Labimethyl, Macasa, Spain) at concentration of 0.3 and 3%, respectively, were used as standards. Micro-organisms were applied 3 days and 6 hours before infection in order to guarantee their establishment on the phyllosphere. The friendly control agents (FCAs) were applied at the same times in order to allow the activity of potential induced resistance. Water was used in the untreated control. Copper hydroxide (Kocide, Du Pont De Nemours) was used at 5 g/l plus neem extract (Neemgard; Thermo trilogy, USA) at 2% were applied as reference in the trial of BCAs and FCAs combination.

Disease severity (percentage of plant area with symptoms) was evaluated from disease onset until the untreated control reached c. 80% severity. Area under the disease progress curve (AUDPC) was calculated for this period (three weeks). Five plant replicates were used for each treatment. Data were analysed with ANOVA using Statistica 7.0 software (Statsoft, Italy) on arcsin transformed AUDPC data. Means were separated with Fisher’s test at $P<0.05$.

### Results

Some BCAs (T39, B69 and Y2) and FCAs (Elot-vis and Chitoplant) reduced late blight (Tables 1 and 2).
Table 1. Efficacy of BCAs against *P. infestans* on tomato under controlled conditions

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>AUDPC*</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>63.7 cd</td>
<td></td>
</tr>
<tr>
<td>T39</td>
<td>26.1 a</td>
<td>0.59</td>
</tr>
<tr>
<td>Y13</td>
<td>72.6 cd</td>
<td>-0.14</td>
</tr>
<tr>
<td>Y89</td>
<td>57.6 bc</td>
<td>0.10</td>
</tr>
<tr>
<td>Y16</td>
<td>69.1 cd</td>
<td>-0.08</td>
</tr>
<tr>
<td>Y2</td>
<td>36.7 ab</td>
<td>0.42</td>
</tr>
<tr>
<td>B69</td>
<td>24.4 a</td>
<td>0.62</td>
</tr>
<tr>
<td>B71</td>
<td>66.6 cd</td>
<td>-0.04</td>
</tr>
<tr>
<td>B19</td>
<td>50.0 bc</td>
<td>0.22</td>
</tr>
<tr>
<td>B6</td>
<td>73.3 cd</td>
<td>-0.15</td>
</tr>
<tr>
<td>F77</td>
<td>100.4 d</td>
<td>-0.57</td>
</tr>
</tbody>
</table>

BCAs were sprayed 6 days and 3 hours before artificial inoculation. AUDPC was calculated from disease severity data collected 12, 16 and 20 days after inoculation.

* Columns with same letter do not differ at $P < 0.05$ (Fisher’s test).

Table 2. Efficacy of FCAs against *P. infestans* on tomato under controlled conditions

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Commercial product</th>
<th>AUDPC*</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Untreated control</td>
<td>188.4 d</td>
<td></td>
</tr>
<tr>
<td>Fatty acids</td>
<td>Tecnobiol</td>
<td>123.7 cd</td>
<td>34.3</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Chitoplant</td>
<td>0.1 a</td>
<td>99.9</td>
</tr>
<tr>
<td>Plant extract</td>
<td>Elot-vis</td>
<td>26.1 a</td>
<td>86.2</td>
</tr>
<tr>
<td>Copper</td>
<td>Labimethyl</td>
<td>73.6 bc</td>
<td>60.9</td>
</tr>
<tr>
<td>Copper</td>
<td>Labicuper</td>
<td>55.6 b</td>
<td>70.5</td>
</tr>
</tbody>
</table>

FCAs were sprayed 6 days and 3 hours before artificial inoculation. AUDPC was calculated from disease severity data collected 12, 16 and 20 days after inoculation.

* Columns with same letter do not differ at $P < 0.05$ (Fisher’s test).

A synergistic effect against *P. infestans* was obtained on tomato plants only with the combination of B19 and Elot-vis. Even if T39 and B69 activity against *P. infestans* was increased, respectively, by the addition of Chitosan and Chitosan and Elot-vis, the efficacy of the two FCAs was reduced when combine with T39, B69 and Y2 (Fig. 1). With high level of AUDPC some inconsistency in the efficacy of Y2 and B69 has to be underlined. Consistent results obtained with Chitoplant, Elot-vis and T39 could be related to the fact that they are formulated products or the result of a more effective mode of action.

Some FCAs and BCAs may have potential for late blight control, but further information is needed. Identification of BCAs or FCAs represents only the first step in the development of effective biological control. Its implementation and optimization needs more knowledge regarding mechanism of action, ecological characteristic and interactions with microbial communities. Through an understanding of these characteristic, we can establish the limitations as well the full potential for biocontrol within this patho-system, and develop strategies for its implementation and management. (Larkin & Fravel, 1998).
Figure 1. Effect of BCAs and FCAs used alone and in combination on late blight AUDPC on tomato plants. Columns with same letter do not differ with $P \leq 0.05$ (Fisher’s test).

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References


Compatibility of *Trichoderma koningii* with chemical fungicides

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**Abstract:** Isolate Th003 of *T. koningii* with high biocontrol activity against different pathogens on tomato plants was tested *in vitro* and *in vivo* for compatibility with routinely used fungicides: chlorothalonil, difenoconazole, methyl-tiofanate, carbendazim, benomyl, copper oxichloride and sulfur. When *in vitro* compatibility tests were conducted, a negative effect of chlorothalonil, difenoconazole, methyl-tiofanate, carbendazim, benomyl, and copper oxichloride expressed as *T. koningii* complete inhibition of germination and growth was observed. Sulfur did not affect these parameters. However, when the effect of the fungicides was evaluated *in vivo*, *T. koningii* was able to germinate, although a significant reduction effect ranging from 10-17% was observed, as compared with the untreated control (*P*<0.0001) with the fungicides carbendazim, benomyl, chlorothalonil and copper oxichloride when germination was evaluated 24 and 48 h after fungicide application. This study highlights the importance of testing biocontrol agents *in vivo* to establish compatibility with chemical pesticides.

**Keywords:** Tomato

**Introduction**

The development of methods for controlling pathogens in different agricultural crops may require adoption of integrated pest management (IPM) strategies (Whipps & Lumsden, 1991) including the use of biological control agents (BCAs). These microorganisms are normally integrated with chemical pesticides necessary for managing crop pests. This means that biologicals will need to be compatible with chemical control agents. In previous works, a native strain of *Trichoderma koningii* (Th003) was selected for its high biocontrol activity against different soil-borne and foliar pathogens, such as *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium splendens* (Cotes et al., 1996), *Botrytis cinerea* and *Oidium lycopersici* (Moreno, 2003). Two biopesticides prototypes were developed with this strain, and formulated as dispersible granules and as wettable powder. This study was undertaken to test compatibility of Th003 with commonly used fungicides with a view to future integration with the BCA.

**Materials and methods**

**In vitro fungicide sensitivity assessment**

The fungicides chlorothalonil, difenoconazole, methyl-tiofanate, carbendazim, benomyl, copper oxichloride and sulfur were tested at the recommended dose (Table 1), half and a quarter of that concentration. All products at different doses were added as aqueous suspensions or solutions to Saboureaud Sacarine Agar (SSA) at 50°C after autoclaving. 100 µL of a *T. koningii* Th003 conidia suspension (1×10⁶ conidia.mL⁻¹) were inoculated on Petri dishes containing SSA with each fungicide and incubated for 16 h at 24°C. When the elongating germ tube was
longer than the conidium diameter (Luz & Fargues, 1997) germination of one hundred conidia was scored by under the microscope. Three replicate tests were set up for each fungicide. Control treatment consisted on *T. koningii* growing in the absence of fungicides.

Table 1. Fungicides evaluated and their full concentration used

<table>
<thead>
<tr>
<th>Fungicide</th>
<th>mg.mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbendazim (Ca)</td>
<td>0.250</td>
</tr>
<tr>
<td>Benomyl (Be)</td>
<td>0.350</td>
</tr>
<tr>
<td>Difenoconazole (Di)</td>
<td>0.125</td>
</tr>
<tr>
<td>Cupper oxichloride (Ox)</td>
<td>12.600</td>
</tr>
<tr>
<td>Clorotalonil (Cl)</td>
<td>0.720</td>
</tr>
<tr>
<td>Methyl-tiofanate (Mt)</td>
<td>0.700</td>
</tr>
<tr>
<td>Sulfur (Su)</td>
<td>2.520</td>
</tr>
</tbody>
</table>

**In vivo fungicide sensitivity assessment**

A greenhouse experiment was conducted using 2-months-old tomato plants grown in soil contained in plastic bags. Treatments were assigned in a completely randomized block design, with plots consisting on 5 tomato plants per treatment. Before BCA application leaves were washed once with sodium hypochlorite 1% and two times with sterile water. Subsequently tomato leaflets were sprayed two times with a conidia suspension of $1 \times 10^6$ conidia.mL⁻¹ by using a volume of 5 mL per leaflet. Immediately after fungus application and eight days after, chemical pesticides were applied at the recommended commercial concentration. One leaflet of each plant was cut at 0, 24 and 48 h after fungicide application (contact time). Leaflets were washed individually in a 250 mL Erlenmeyer with 50 mL of Tween 80 0.1% (150 rpm, 15 min) and samples of 1mL suspension were inoculated on Petri dishes with Malt Extract Agar (MA). Conidia germination was assessed as mentioned above, each treatment consisted on 15 replicates.

**Results and discussion**

**In vitro fungicide sensitivity**

The results summarized in Fig. 1 show that *T. koningii* germination was significantly affected ($P<0.0001$) by the fungicides benomyl, difenoconazole, copper oxichloride and clorothalonil at the three concentrations. Germination with these fungicides was less than 5% while the without fungicide conidia germination reached 98%. Germination was not significantly reduced by the fungicides methyl-tiofanate and sulfur, except in the treatment where 100% of methyl-tiofanate recommended dose was assayed. Sensitivity of *T. koningii* to carbendazim was moderat; significant differences ($P<0.0001$) were found between the germination in presence of this fungicide and the control treatment. However, germination with the three evaluated doses of carbendazim varied between 80-84%, while with some fungicides germination was less than 5%. Results suggest that *T. koningii* was not tolerant to the fungicides benomyl, difenoconazole, copper oxichloride and clorothalonil after exposure in culture medium. This could be explained by the mode of action of these fungicides consisting mainly in the inhibition of DNA synthesis (De Liñan, 1997), important process during conidia germination.

Similarly Sosa et al. (2003), observed a significant reduction in *Nomuraea rileyi* germination when the fungus was grown in presence of benomyl and difenoconazole and a no
effect on germination with the fungicide carbendazim. Kosoki et al. (2001) also reported a drastic effect of the fungicide clorothalonil on *Colletotrichum acuatum* - 12% maximum value of germination *in vitro*. These authors also described no effect of sulfur and methyl-tiofanate on *C. acuatum* germination as it was observed in the present work for *T. koningii* Th003.

In spite of *T. koningii* Th003 ability to germinate in the presence of sulfur and methyl-tiofanate, morphological changes were observed on germinated conidia (Fig. 2). Conidia and germ tube in presence of these fungicides swollwed with more vacuolization than in the control treatment. These changes may be related with fungicides interference with cellular division allowing fungus germination but affecting germ tube development and generating abnormal cells (De Liñan, 1997). Obtained results let to conclude that *T. koningii* Th003 was moderately tolerant to sulfur and very sensitive to other evaluated fungicides *in vitro*.

**In vivo fungicide sensitivit**

When fungicides were applied immediately after fungus application (contact time=0 h) no significant effect was observed over *T. koningii* germination, resulting in germination higher than 80% (Fig. 3). After 24 h of contact, only methyl-tiofanate significantly reduced germination (62%, \( P<0.0001 \)). Carbendazim, benomyl, difenoconazole, clorothalonil and copper oxichloride showed a significantly effect over conidia germination after 48 hours of contact (\( P<0.0001 \)), with germination values of 60-80%. It can be concluded that germination of *T. koningii* is affected by most of the evaluated fungicides when contact time between BCA and fungicides is more than 24 h. However the effect is not as dramatic as observed *in vitro*.

When the fungicides were applied 8 days after *T. koningii* application, germination in all treatments and the control was reduced as compared with immediate fungicide-fungus application; this reduction was probably due to the length of incubation and the atmospheric conditions, which could cause latency in conidia (Elad & Kirshner, 1993). Contact time effect presented the same behaviour obtained when fungicides were applied immediately after fungus application (Fig. 4). The results obtained suggest a moderate compatibility of *T. koningii* Th003 with the evaluated fungicides under *in vivo* conditions.
Conidia germination under \textit{in vivo} study was significantly higher than the \textit{in vitro} exposure as reported by other authors (Budge & Whipps, 2000; Frabel et al., 2005; Kubilay & GökSe, 2004). No relationship between the effect \textit{in vitro} and \textit{in vivo} was observed for the tested fungicides. These results demonstrated the importance of assessing pesticides compatibility \textit{in vivo}. The combination of different control methods can offer an advantage over one treatment (from this research), it is evident that some combinations of fungicides with \textit{T. koningii} could be possible and may lead to their combined use in some IPM programmes.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{In vivo effect of fungicides on \textit{T. koningii} germination (assessed after 24h) when they were applied immediately after BCA. Fungicide contact time 0 hours, fungicide contact time 24 hours, fungicide contact time 48 hours. Bars represent the standard deviation.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{In vivo effect of fungicides on \textit{T. koningii} germination (assessed after 24h) when they were applied 8 days after BCA. Fungicide contact time 0 hours, fungicide contact time 24 hours, fungicide contact time 48 hours. Bars represent the standard deviation.}
\end{figure}

\section*{Acknowledgements}

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Biocontrol strategy in tomato soil-less culture by combining slow filtration and *Pythium oligandrum* inoculation

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**Abstract:** *Pythium oligandrum* is a biocontrol agent which has been successfully used to protect plants from root diseases in hydroponic cultures. After introduction of selected strains of *P. oligandrum* in the rhizosphere of tomatoes grown in greenhouse, their persistence was assessed by two molecular methods, DNA macroarray and real-time PCR; and results were compared to those from a plate-counting method. PCR-based methods detected *P. oligandrum* throughout the 6-months growing season, whereas plate counting indicated its presence only over the first 3 months. These results provide new data about persistence and inoculation strategies for *P. oligandrum* on plants. In the same experiment, a slow filter (SF) was used to eliminate pathogenic fungi from the nutrient solution. Antagonistic bacteria strains (*Bacillus cereus* or *Pseudomonas putida*) were inoculated in the SF unit to enhance its efficacy. Each month, its ability to eliminate *Pythium* spp. was evaluated by assessing populations found in inflow and outflow water, and in the rhizosphere. It showed a drastic reduction of *Pythium* spp. from the nutrient solution by SF. However, *P. dissotocum* was endemic and was routinely detected in the rhizosphere of inoculated and control plants.

**Key words:** Integrated Pest Management, root rot, oomycetes

**Introduction**

In soilless culture, water supply is the main source of introduction of plant pathogens in greenhouses and recycling of the drained solutions increases the pathological risks due to these water molds. The technique of biofiltration associates mechanical filtration and biological activity of bacterial biofilms. This filtration system has proven to be efficient in the elimination of various pathogens. Compared to a control filter, Deniel et al. (2004) have evidenced that biological activation of columns by antagonistic bacteria enhances very significantly *Fusarium oxysporum* elimination. A previous experiment performed by our group have demonstrated that the combination of slow filtration to disinfect nutrient solutions with the use of antagonistic fungi to colonize and to protect the roots from pathogenic attacks can offer good prospects for minimising pathological problems in soilless cultures (Rey et al., 1999).

In that context, the introduction in the rhizosphere of *P. oligandrum* presents numerous advantages (Le Floch et al., 2003ab). However, an enhanced rhizosphere competence by *P. oligandrum* is a prerequisite for an efficient plant protection strategy. Accurate detection and quantification is a prerequisite for studying rhizosphere ecology and managing microbial community structure for better disease suppressiveness. In the present experiment, culture-
dependent and molecular methods have been used to determine the rhizosphere persistence of *P. oligandrum*. We used a DNA array technology based on PCR amplification of the ITS allowing us to detect more than a hundred *Pythium* species (Tambong et al., 2006). Results were compared with those obtained with real-time PCR. Real-time PCR can detect small quantities of target DNA from complex environmental samples and has been frequently used in studies to assess fungal populations.

The aim of the present study was to use a slow filter biologically activated with antagonistic bacteria and to inoculate *P. oligandrum* in the rhizosphere. Pathogens reduction in nutrient solutions and *P. oligandrum* root colonisation will increase plant protection in tomato soilless culture.

**Material and methods**

**Filter unit**
The filter unit was installed in an experimental greenhouse (CATE, Saint Pol de leon, France) at room temperature. The effluent solution flowed through a column consisting in a plastic pipe (220 cm in length and 40 cm in inner diameter) filled with pozzolana particles. The biological activation of the filter was performed by inoculating the pozzolana (in February and March) with antagonistic bacteria (*3 Pseudomonas putida* and *2 Bacillus cereus* strains). To test the effectiveness of slow filtration, regular samplings were made during the cultural season, from March to September. Each month, 3 samples of nutrient solution were collected just right before it flowed through the filter, 3 others were taken from the filter effluent. Detection of *Pythium* spp. was performed because these are key components of roots and nutrient solution microflora in soilless greenhouses.

**Plant material and Pythium oligandrum inoculations**
Tomato plants, *Lycopersicon esculentum* Mill. cv Tradiro (De Ruiter Graines, France), were grown in coco-fibre slabs (4 plants per slabs) in a commercial greenhouse (136 tomato plants per condition). Plants inoculated with *P. oligandrum* and control plants were totally separated by distinct slow sand filter (SSF) units used to recycling the nutrient solution (Deniel et al., 2004). Inoculation with 3 strains of *P. oligandrum* was performed as previously described using an oospore-mycelium homogenate (Le Floch et al., 2003b).

**Assessment of root colonisation by Pythium spp. using plate counting method**
Roots from control and *P. oligandrum*-inoculated tomato plants were sampled monthly from March to August. For each sample, root colonisation by *Pythium* spp. was assessed through direct plating of non-disinfected root fragments on semi-selective medium and incubated at 25 °C (+/- 1°C) in the dark.

**Evaluation of Pythium spp. root colonisation by DNA array hybridisation**
DNA was extracted in duplicate by using Fast DNA (MP biomedicals, France) procedure with extraction buffer, CLS-VF, required for processing infected plant tissues as described by the manufacturer. Nuclear rDNA region of internal transcribed spacers (ITS) was amplified using a universal primer, and an oomycete-specific primer as described by Tambong et al. (2006). DNA hybridisation and detection on an array of specific oligonucleotides was done as described by Tambong et al. (2006).

**Assessment of root colonisation by Pythium spp. using real-time PCR**
Specific primers and probes were designed and tested for specificity to the target *Pythium* species. Taqman probes were based on oligonucleotides sequences, oli142 and dis183, spotted on the *Pythium* array and on their specificity in hybridizing the targeted species amplicons (Tambong et al., 2006).
The real-time PCR reactions were performed in a Chromo 4 thermo-cycler (Bio-Rad, Mississauga, Ontario, Canada) and were carried out as described by Le Floch et al. (2006).

Results and discussion

Elimination of Pythium spp. contained in nutrient solutions after slow filtration
Compared to a control filter, the filtration efficacy was enhanced further to a biological activation of columns by inoculating the pozzolana grains contained in the filtering unit with either P. putida or B. cereus selected strains. Pythium spp. elimination rates were high and in the range of 93.9-100%, the lowest value (82.4) was recorded in April for P. putida-amended filter. Compared to control, inoculated bacteria-filters show very good efficacy and stability over time.

Root colonisation by Pythium spp. using plate counting, DNA array hybridisation and Real-Time PCR
Most Pythium spp. isolates excluding P. oligandrum were fast-growing with no reproductive sexual organs on a culture medium that would normally favour their production. The prevalent type was strictly filamentous and was identified as Pythium group F on morphological characters. Using DNA array hybridisation, P. dissotocum was almost the only species detected on roots of plants, inoculated or not with the antagonist. It allowed us to draw a time course of root colonisation by P. dissotocum based on hybridisation intensities of the specific oligonucleotide, dis183. No significant differences by real-time PCR were found in the amount of DNA detected in inoculated and non-inoculated plants. A delay in the detection of P. dissotocum in the P. oligandrum-inoculated plants was observed during the first two months of the growing season compared to control plants. Increasing P. dissotocum DNA quantities were observed throughout the season in both treatments. Other Pythium species (P. ultimum, P. sylvaticum and P. intermedium) were sporadically detected, but none exhibited hybridisation signals as intense as P. dissotocum.

Assessment of the persistence of P. oligandrum by plate counting, DNA array hybridisation and Real Time-PCR
Significant differences were observed on the dynamics of root colonisation by P. oligandrum as detected by culture-dependent or molecular method. DNA array hybridisation and real-time PCR detected P. oligandrum throughout the 6-months growing season, whereas plate counting indicated its presence only the first 3 months. This difference could be due to the detection limit of the technique. It is highly probable that during the first 3 months of the experiment, P. oligandrum which was the most frequent and abundant Pythium species on inoculated roots can readily grow since no competition with other Pythium spp. occurred in selective medium. In the last 3 months of the growing period, a dramatic increase in P. dissotocum populations was detected in the rhizosphere. It is likely that this fast growing fungus can compete with P. oligandrum and prevents its development on selective culture medium.

P. oligandrum is an aggressive mycoparasite of pathogenic Pythium species and this study confirmed its aptitude to colonise roots infected by pathogens. It should be noted that P. oligandrum seems to have a slight influence on the distribution of other Pythium species colonising the roots. This relatively low diversity of Pythium species confirms results of previous studies conducted in soil-less cultures. This is likely due to the use of an efficient filtration system to eliminate Pythium spp. and other fungi from recycled nutrient solutions. In conclusion, the combination of biologically activated-slow filtration to disinfect nutrient solutions with the use of P. oligandrum to colonise and protect the roots from pathogenic
attacks can offer good prospects for minimising or even preventing problems associated with pathogenic microorganisms in soil-less cultures.

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Control of citrus black spot (Guignardia citricarpa) by biological control agents and other alternative products

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Abstract: Citrus black spot (CBS) is responsible for substantial damage in citrus, in several countries. In Brazil, this disease occurs in several municipalities in the State of São Paulo, in an area that is highly representative of the state’s citriculture. Black spot control basically relies on the use of protective or systemic fungicides, applied at 28-day intervals. The objective of this work was to evaluate the effects of biocontrol agents (Bacillus subtilis and Trichoderma sp.) and other alternative products (cow milk and biofertilizer) to control CBS in organic and conventional systems. In the first experiment, the following treatments were done on ‘Pera’ plants: B. subtilis (10^7 and 10^8 CFU ml^-1); autoclaved Milhocina (0.5%) + Molasses (0.5%); Trichoderma sp. (10^6 conidia ml^-1); cow milk (5%) and Microgeo® (commercial biofertilizer currently used by citrus organic growers). The severity of the disease on 50 fruits at harvest stage collected randomly from each replication plant were evaluated by means of a six-category scale, which 1=0.5%, and 6=49% of fruit area with lesions. The percentage of fruits classified at class 1, 2 and 3 to 6 were calculated. The milk and B. subtilis (10^8) treatments did not differed significantly from each other and presented the higher percentage of fruits classified at class 1 (26 and 19%, respectively) and the lower percentage of fruits at class 3 to 6 (30 and 36%, respectively). These treatments were significantly superior to Microgeo® treatment (11, 38 and 51%, respectively for fruits at class 1, 2 and 3 to 6). In the second experiment, in a conventional ‘Valencia’ orchard, different doses (0, 2.5, 5, 7.5 and 10% v/v) of a biofertilizer (produced by aerobic fermentation of a mixture of molasses, compost cattle manure, earthworm humus, yeast and water) were sprayed and compared with a standard fungicide treatment. The percentage of fruits classified as 1 and 2 were 4.4, 8.0, 5.8, 6.6, 4.9 and 12.8, respectively for the treatments 0, 2.5, 5.0, 7.5 and 10% v/v of a biofertilizer (produced by aerobic fermentation of a mixture of molasses, compost cattle manure, earthworm humus, yeast and water) sprayed and compared with a standard fungicide. The percentage of fruits classified as 5 and 6, for the same treatments were 38, 29, 28, 28, 28 and 19. These results indicate the potential of the use of biofertilizer, milk and B. subtilis as alternatives for citrus black spot control, especially in organic orchards.

Key Words: alternative control, biological control

Introduction

Citrus black spot (CBS), caused by Guignardia citricarpa (anamorphic stage: Phyllosticta citricarpa), is responsible for substantial damage in citrus, in several countries of Africa, Asia, South America and Oceania. In Brazil, this disease occurs in several municipalities in the State of São Paulo, in an area that is highly representative of the state’s citriculture. The disease also occurs in the states of Rio de Janeiro and Rio Grande do Sul, where it has caused extensive losses in the last two decades. Black spot control relies on the use of protective fungicides, applied at 28 days intervals, or on a mixture of protective + systemic fungicides + mineral or vegetable oil (Schutte et al., 1997).

The problems associated with the intensive use of fungicides had raised interest in the development of alternative techniques aimed at agricultural sustainability. Kupper et al. (2006) verified the effect of biofertilizer for the control of citrus black spot. Moretto (2000)
and Bettiol et al. (2005) verified the potential of *Bacillus subtilis* and *Trichoderma* isolates in the control of citrus post-bloom fruit drop, caused by *Colletotrichum acutatum*, and *G. citricarpa*. The objective of this work was to evaluate the effects of biocontrol agents (*Bacillus subtilis* and *Trichoderma* sp.) and other alternative products (cow milk and biofertilizer) to control citrus black spot in organic and conventional systems.

**Material and methods**

The first experiment was conducted in a ‘Pera’ organic orchard. The following treatments were done: *B. subtilis* (10^7 and 10^8 CFU ml\(^{-1}\)); autoclaved Milhocina (0.5%) + Molasses (0.5%); *Trichoderma* sp. (10^6 conidia ml\(^{-1}\)); cow milk (5%) and Microgeo® (commercial biofertilizer currently used by citrus organic growers). All treatments, except Microgeo®, were sprayed at scheduled intervals (0, 28, 56, 84, 112, 140 and 168 days) from December 8, 2004 (bloom period) to August 28, 2005 (fruit harvest). The Microgeo® treatment was sprayed along all year at a monthly interval. The experiment was conducted in a completely randomized blocks design with 5 treatments and 15 replication plants. The severity of the disease on 50 fruits at harvest stage collected randomly from each replication plant were evaluated by means of a six-category scale, which 1=0.5% and 6=49% of fruit area with lesions. The percentage of fruits classified at class 1, 2 and 3 to 6 were calculated.

In the second experiment, in a ‘Valencia’ conventional orchard, different doses (0, 2.5, 5, 7.5 and 10% v/v) of a biofertilizer (produced by aerobic fermentation of a mixture of molasses, compost cattle manure, earthworm humus, yeast and water) were sprayed at the same dates as the first experiment and compared with a standard fungicide treatment.

**Results and discussion**

The milk and *B. subtilis* (10^8) treatments did not differed significantly from each other and presented the higher percentage of fruits classified at class 1 (26.3 and 19.4%, respectively) and the lower percentage of fruits at class 3 to 6 (30 and 36%, respectively) (Fig. 1). These treatments were significantly superior to Microgeo® treatment (11, 38 and 51%, respectively for fruits at class 1, 2 and 3 to 6) (Fig. 1). Trees of citrus sprayed with *B. subtilis* (10^8) and milk (5%) presented the lower indexes of disease in the fruits (0.36), when compared with others (Fig. 2). The *Trichoderma* sp. was ineffective in reduce the disease and present disease index (0.47) superior to the standard treatment Microgeo® (0.43) (Fig. 2).

In the second experiment, in a conventional orchard, all the treatments reduced the severity of the disease. Significant differences between the different doses of biofertilizer were not observed. The fungicide treatment presented the lower index of disease when compared to the others (Fig. 3). The percentages of fruits classified as 1 and 2 were 4.4, 8.0, 5.8, 6.6, 4.9 and 12.8, respectively, for the treatments 0, 2.5, 5.0, 7.5 and 10% of biofertilizer and fungicide. The percentage of fruits classified as 5 and 6, for the same treatments were 38, 29, 28, 28, 28 and 19. These results indicate the potential of the use of biofertilizer, milk and *B. subtilis* as alternatives for citrus black spot control, especially in organic agriculture.
Figure 1. Effect of milk, *B. subtilis*, *Trichoderma* and biofertilizer (Microgeo®) on the occurrence of citrus black spot, caused by *G. citricarpa*, in 'Pera' orange trees, during the 2004-2005 season.

Figure 2. Effect of milk, *B. subtilis*, *Trichoderma* and biofertilizer (Microgeo®) in the indices of citrus black spot, caused by *G. citricarpa*, in 'Pera' orange trees, during the 2004-2005 season.
Figure 3. Effect of biofertilizer in the indices of citrus black spot, caused by *G. citricarpa*, in 'Valencia' orange trees, during the 2004-2005

Figure 4. Effect of biofertilizer on occurrence of citrus black spot, caused by *G. citricarpa*, in 'Valencia' orange trees, during the 2004-2005

References


Survival of *Trichoderma harzianum* T22 in soil after chloropicrin fumigation

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**Abstract**: Grapevine root rot caused by *Armillaria mellea* is an important disease in some areas of northern Italy (Trentino). Chemical fungicides are ineffective in controlling the disease and biocontrol agents (BCAs) show only partial and insufficient protection. In autumn 2005, the efficacy of the fumigant chloropicrin was tested against *A. mellea* in two different fields. After the fumigation, the soil was treated with *Trichoderma harzianum* strain T22 to evaluate its survival and development. The aim was to evaluate if chloropicrin fumigation can reduce the inoculum and favour the establishment of the BCA by reducing competition with the indigenous microflora. After burying samples of *A. mellea* at different depths, the soil was treated with chloropicrin and covered with a plastic film. Part of the area was left untreated and similar *A. mellea* samples were buried in it. Ten days after fumigation, *A. mellea* samples were removed from soil to evaluate their viability. At the same time, portions of the previously fumigated soil and of the untreated part were treated with a water suspension of *T. harzianum* T22 conidia. Soil samples were collected at different times. For each sample the recovered *Trichoderma* spp. CFUs were counted on selective media. The fumigation killed *A. mellea* samples placed in the soil. The residual chloropicrin present after fumigation also reduced the viability of *Trichoderma* T22 that was spread in the soil after the removal of the plastic film. In fact less *Trichoderma* spp. colonies were measured one week after the application in the chloropicrin treated area compared to the untreated one.

**Key words**: *Armillaria* spp.

**Introduction**

Grapevine root rot caused by *Armillaria mellea* is an important disease in some areas of northern Italy (Trentino). Chemical fungicides are ineffective in controlling the disease and biocontrol agents (BCAs) showed only a partial protection. This could be due to the inability of chemicals to reach the *A. mellea* colonised root tissue, the aggressiveness of the pathogen, the presence of high inoculum levels in infected soils and the competition of natural microflora with the introduced BCA.

Since the use of methyl bromide is restricted because of its deleterious effects on stratospheric ozone concentrations, several products are being considered as possible replacements; among these, chloropicrin is currently used against soil-borne pathogens (Ruzo, 2006). Chloropicrin is a liquid fumigant at room temperature with moderate vapour pressure and boiling point (Ruzo, 2006). Typically, it is injected into the soil 20-25 cm below the surface and moves rapidly by diffusion within 30 cm of injection, but may diffuse to a maximum depth of 120 cm in sandiest soils (Ruzo, 2006). *T. harzianum* strain T22 is an antagonist with specific competitive effect for space and nutrients against soil-borne pathogens. *Trichoderma* spp. are more resistant to the fumigant than *A. mellea*, have few
competitors and can reproduce rapidly. Thus the antagonist may build up very quickly in fumigated soils (Munnecke et al., 1981).

The aim of this experiment was to understand if chloropicrin fumigations effect on *A. mellea* inoculum and favour the establishment of a BCA as *T. harzianum* strain T22, by evaluating its survival after fumigation.

**Material and methods**

In 20 October 2005, the efficacy against *A. mellea* of chloropicrin fumigations and their side effects on *T. harzianum* T22 were tested in two different locations in Lagosanto (Ferrara Province, northern Italy) named soil 1 and soil 2. In each soil, samples of *A. mellea* colonies grown for 15 days on Malt Extract Agar (MEA) were buried at different depths: 25 samples were buried at 20 cm and 25 samples at 40 cm. Identical 25 *A. mellea* samples were buried only at 40 cm depth since from previous experiments no differences were seen in survival of *A. mellea* at different depths in the same soil. The soil was treated with chloropicrin (1.6 g/l) and covered with a plastic film.

Ten days after fumigation when plastic film was removed, *A. mellea* samples were collected, surface sterilized with sodium hypochlorite (1%) and transferred onto sterile MEA medium to evaluate their viability: growth of new mycelium on the agar was assessed after 15 days of incubation at 25°C.

At the same time of plastic removal, the surface of previously fumigated and non fumigated soil was treated with a water suspension (10 liters/m²) of *T. harzianum* T22 conidia (0.3 g/l). Soil samples (3 replicates) were collected at different times at 10 cm depth: at the removal of the plastic film (before the treatment with *T. harzianum* T22), 30 minutes, one week and seven months (during spring) after the treatment with *T. harzianum* T22. In the same moments, soil samples (3 replicates) were collected from non fumigated and *T. harzianum* T22 treated areas. For each sample the recovered *Trichoderma* sp. CFUs were counted on selective media (potato dextrose agar, rose Bengal 50 ppm, chloramphenicol 100 ppm, streptomycin 50 ppm): 1 g of soil was subjected to successive dilutions in sterile water (dilutions of 100, 1000, 10000 times), then 1 ml of the suspension was put in Petri dishes with the selective media to count *Trichoderma* spp. CFUs. Three repetitions for each dilution were used.

**Results and discussion**

**Efficacy of chloropicrin fumigation against *A. mellea* in soil**

After 15 days of incubation, none of the chloropicrin treated *A. mellea* samples from the two sites were alive after fumigation, instead mostly of the untreated ones were vital (Table 1). In the chloropicrin treated samples was not observed either the development of other micro-organisms while a lot of other fungi and bacteria developed on untreated *Armillaria* samples.

**Effect of chloropicrin on *T. harzianum* survival in the soil**

After the fumigation and before the treatment with *T. harzianum*, no *Trichoderma* spp. CFU/g soil developed from samples collected in the soil 1 (Table 2). The average presence of *Trichoderma* spp. in non-fumigated *T. harzianum* T22 untreated soil was 100 CFU/g. This number represents the natural *Trichoderma* spp. population present in the soil, since the used media is selective for all the *Trichoderma* genus and was seen also in not fumigated and not treated soil samples collected at one week and seven month.
Table 1. Percentage of *A. mellea* samples, removed at different depths after Chloropicrin fumigation in treated and untreated areas, showing new mycelium growth on medium

<table>
<thead>
<tr>
<th></th>
<th>Chloropicrin treated</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 cm</td>
<td>40 cm</td>
</tr>
<tr>
<td>Soil 1</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>Soil 2</td>
<td>0 a</td>
<td>0 a</td>
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</tbody>
</table>

*aFor each soil, treatment and depth 25 *A. mellea* samples were used. Different letters in the same row represent significant differences at the $\chi^2$ test for $P<0.01$.

In soil 1 samples collected immediately (30 minutes) after the treatment with *T. harzianum* T22 in the fumigated areas, an average of 1350 CFU/g soil was found, but one week after treatment the number decreased to 415 CFU/g soil. In non fumigated areas of soil 1, immediately and one week after *T. harzianum* T22 application, respectively 1310 and 1250 CFU/g were found. In samples collected in the spring 2006 (seven months after *T. harzianum* treatment) the average number of *Trichoderma* spp. CFU/g was relatively stable respect the previous autumn assessment with a slight increase of *Trichoderma* spp. colonies in fumigated soil and a slight, but not statistically significant, decrease in not fumigated one. The significant decrease of CFU/g soil one week after T22 treatment in the fumigated demonstrate that the residual chloropicrin present at the end of the fumigation can affect a bio control agents applied immediately after. The unchanged *Trichoderma* spp. presence between the two assessments done before and after winter, in chloropicrin and untreated soils demonstrate that the presence of *Trichoderma* spp. (likely *T. harzianum* T22) is stable during winter and the organism can survive for at least seven month after application, but does not increase in the chloropicrin treated soil (Table 2). A similar chloropicrin effect on *Trichoderma* spp. survival was obtained in the second location (soil 2).

Table 2. Average *Trichoderma* spp. colonies (CFU/g soil) developed on selective media at different sampling times, in chloropicrin fumigated and untreated (not fumigated) soil 1

<table>
<thead>
<tr>
<th></th>
<th>Chloropicrin fumigated</th>
<th>Not fumigated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>End of chloropicrin fumigation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min after <em>T. harzianum</em> T22</td>
<td>1350 a</td>
<td>1310 a</td>
</tr>
<tr>
<td>1 week after <em>T. harzianum</em> T22</td>
<td>415 b</td>
<td>1250 a</td>
</tr>
<tr>
<td>7 month after <em>T. harzianum</em> T22</td>
<td>433 b</td>
<td>1225 a</td>
</tr>
</tbody>
</table>

Different letters in the same column represent significant differences, with the Kruskal-Wallis non parametric test at $P<0.05$.

Chloropicrin fumigation was able to kill the *A. mellea* samples placed in the soil. This encouraging results suggest that the fumigant is effective against the fungus when it is present freely in the soil, but the growth of *A. mellea* inside the root tissue may reduce this activity. The residual chloropicrin after fumigation, or the partial degradation of active ingredient,
reduced the viability of *Trichoderma* spp. applied to the soil after the removal of the plastic films. The application of a BCA immediately after the end of chloropicrin fumigation is therefore not suggested. Seven months after the treatment the number of *Trichoderma* spp. colonies in fumigated and not fumigated soil was stable. This result proves that *T. harzianum* T22 survives very well in the soil, but the population does not increase during time. Chloropicrin has no effect in promoting the development of *T. harzianum* T22. Longer time and several applications of the fumigant and the antagonist are probably necessary to achieve a significant increase in *Trichoderma* population, as described by de lo Santos et al. (2003).

Although further experiments are needed to prove control efficacy of *A. mellea* grapevine root rot under field conditions, chloropicrin seems to be valuable tool, but it is not useful to increase the population of a potential *A. mellea* BCA such as *T. harzianum* T22.

**Acknowledgements**

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**References**


Enhancement of *Pantoea agglomerans* CPA-2 by the combination with curing to control post-harvest diseases on oranges

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Abstract: Green and blue mould, caused by *Penicillium digitatum* and *P. italicum*, respectively, are response of serious economic losses in citrus packinghouses. Curing, by holding fruit at high temperatures and humidity, to enhance host defence mechanisms, is an attractive commercial possibility for control postharvest decay. The biological control agent *Pantoea agglomerans* (CPA-2), isolated from apple surface, is an effective antagonist to the major post-harvest pathogens on pome and citrus fruits. The present study focused the combination of “short” or “long” curing and *Pantoea agglomerans* (CPA-2).

In “short” curing assays, all oranges were previously wounded and inoculated with *P. digitatum* or *P. italicum* at 10^6 conidia/ml. After air-drying oranges were treated: (i) with *P. agglomerans* (CPA-2) at 2×10^8 CFU/ml, (ii) cured at 55°C for 4 h, (iii) with biocontrol agent after the curing treatment. Control was non treated fruits. To evaluate the effect of “long” curing, fruits were wounded as previously described. After 24 h of infection establishment, fruits were treated as above, except that the in curing treatments fruits were cured at 40°C for 18 h followed for 6 h at 20°C. Seven days after storage at room temperature, all treatments successfully controlled blue and green mould, in both assays. However the best control decay was achieved combining curing with biocontrol agent. The combination of *P. agglomerans* with curing at 55°C for 4 h reduced the development of *P. digitatum* and *P. italicum* by 60% and 52%, respectively, with long curing at lower temperature, the reduction was 80% for *P. digitatum* and 82% for *P. italicum*.

From the present results it is concluded that for the reduction of postharvest decay of both pathogens the combination of “short” or “long” curing with *P. agglomerans* (CPA-2) improve the biocontrol activity of the antagonist. However in a commercial point of view the a curing treatment at 40°C for 18 h integrated with *P. agglomerans* is more effective, with no effects in fruit quality, such as weight loss, and for packinghouses will be easily to reach lower temperatures in a commercial chamber.
Integrated management of soil-borne pathogens as a tool for prolonged use of rockwool substrate for tomato growing in an open hydroponic system

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Abstract: Two spring and two autumn trials with greenhouse tomatoes were conducted, in which preplanting chemical disinfestation of two- and three-year old rockwool slabs with a disinfectant containing dimethylalkylbenzyl ammoniumchloride and polyhexamethylene biguanidine-hydrochloride (at 1500 ppm) was integrated with a complementary postplanting application of biocontrol agents (*Pythium oligandrum*, *Trichoderma viride* B35) or a mixture of fungicides (propamocarb + tiophanate-methyl). Additional applications of *P. oligandrum*, *T. viride* B35 and the fungicide mixture increased the marketable yield in comparison with the disinfectant alone, on the average by 3.2, 4.0 and 4.9% in the spring crops and by 11.8, 2.1 and 6.9% in the autumn crops, respectively.

Key words: chemical disinfestation, greenhouse, propamocarb, *Pythium oligandrum*, re-used rock-wool, tiophanate-methyl, *Trichoderma viride*

Introduction

Rockwool has been the most often used artificial substrate in different soilless cultivation systems of greenhouse vegetables. Although rockwool slabs can be re-used, in most instances commercial growers use cheaper single-year rockwool and the slabs are discarded after one growing season (van Os, 2001). Such a procedure prevents the next crop from transmitting soil-borne diseases and enables to avoid problems with disinfestation. Hochmuth & Hochmuth (1998) do not recommend re-using of old rockwool slabs and they claim that simple disinfestation might probably not be sufficient, unless steaming is done, and that some attempts in the USA to re-use rockwool slabs have not been successful. On the contrary, it was shown that the yield of greenhouse tomatoes in the second year of growing in the same rockwool could be even significantly higher than that in the first year (Wysocka-Owczarek, 1989). An additional reason justifying the reuse of rockwool slabs seems to be connected with the possibility of disease decline in soilless systems with a continuous cropping, due to build-up of suppressiveness to some soil-borne pathogens (Postma, 2004).

The aim of the present study was to evaluate if integration of a chemical disinfestation of two- or three-year old rockwool slabs with introduction of biocontrol agents *Pythium oligandrum* and *Trichoderma viride*, or with additional drenches of the plants with fungicides, increases root health status and productivity of greenhouse tomato plants.

Material and methods

The greenhouse experiments with tomato plants, grown as spring and autumn crops on two- and three-year-old rockwool slabs in an open system, were conducted for two consecutive
years in the presence of *Phytophthora nicotianae* var. *nicotianae* (*Pnn*) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*) in the root zone. The pathogens in the substrate were present from the artificial inoculations done in the previous years.

Four days before planting, the slabs were treated with a commercial disinfectant (Desogerme SP), containing dimethylalkykybenzyl ammoniumchloride and polyhexamethylene biguanidine-hydrochloride (QAC+Chx), using a volume of disinfectant solution sufficient for full saturation of the rockwool. The disinfectant was used at a rate enabling to attain a concentration of 1500 ppm in the whole volume of the rockwool slabs. After an exposure of 24 h, each slab was leached with about 15 L of the nutrient solution.

*T. viride* B35, strain resistant to benomyl and iprodione, was introduced at planting time and 6 weeks later, using 100 ml of the conidial suspension containing $4.2 \times 10^5$ cfu·ml$^{-1}$ per plant. Plant drenches with 50 ml of 0.05% suspension of *Pythium oligandrum* (Polyversum) were applied at the same time as *T. viride*. Plants treated with fungicides were drenched with 300 ml of a mixture of tiophanate-methyl (25 mg a.i. per plant) and propamocarb (60 mg a.i. per plant) one and three weeks after planting.

The experimental design consisted of five integrated phytosanitary treatments in a factorial combination with two batches of re-used rockwool slabs (two- and three-year old), replicated four times. One slab (100×20×7.5 cm) with three tomato plants constituted an individual plot. In all trials the long shelf life tomato cultivar ‘Graziella’ was grown. The tomato plants in spring and autumn trials were grown to 7 and 5 clusters, respectively. The plants were managed according to standard cultural recommendations for tomato cultivation on rockwool.

After the last harvest the severity of root rotting as well as the internal and external disease symptoms in the crown area were evaluated separately according to a 0-5 scale. All data were subjected to analysis of variance and means were compared using Newman-Keuls test ($P=0.05$).

**Results and discussion**

In 2001 spring trial, additional treatment of plants grown in disinfected rockwool with an antagonistic fungus *T. viride* B35 or a mixture of propamocarb and thiophanate-methyl, regardless the time of using the substrate, significantly increased fruit yield in comparison with the control. The disinfectant QAC+Chx used alone or combined with a biocontrol agent containing *P. oligandrum* did not influence significantly the marketable yield. In the second year of the experiment, however, the phyto-sanitary treatments did not have significant influence on the fruit yield.

In 2002 at the end of spring growing cycle, the intensity of *Phytophthora* crown and root rot disease was visibly higher than in 2001, but in both years of experiments, no significant differences between treated and control plots were noted. Moreover, time of using rockwool did not influence the severity of the disease symptoms.
The measurements of plant height (24 days after planting) and fresh weight of leaves removed from the first cluster indicate that, in the two consecutive years of the experiments, chemical disinfection of slabs alone and combined with additional introduction of biocontrol agents or fungicides did not influence significantly the vegetative growth at the beginning of growing season in spring crops (data not shown).

In contrast with spring trial of 2001, a significant interaction between phytosanitary treatments and the period of using rockwool for the marketable yield was found in autumn trial. In two-year-old slabs all phytosanitary treatments influenced the yield similarly, whereas in three-year-old slabs application of the disinfectant combined with *P. oligandrum* was significantly better than application of the disinfectant alone or integrated with *T. viride* B35. The influence of time of using the slabs was significant only in the case of QAC+Chx + *T. viride* B35, where the yield in the fourth cycle was higher than that in the sixth cycle. In the 2002 autumn trial, all treatments of slabs caused visibly smaller increases in the yield that in the previous year. Regardless of the successive growing cycle in the same rockwool slabs, significantly higher fruit yield in comparison with the control was obtained from plants grown in disinfested slabs, with a complimentary application of *P. oligandrum* or a mixture of fungicides.

Figure 1. Influence of an integrated protection of greenhouse tomatoes against soil-borne diseases in rockwool culture on the marketable yield in two- and three-year old slabs in relation to the yield obtained in new slabs in spring and autumn crops.
In 2001 spring crop the yield of control plants grown in slabs used for the second and third year (the third and fifth cycle of growing) was almost the same as that obtained in slabs in the first year of using. In year 2002, the fruit yield in the third and fifth cycle of growing in the same slabs was higher (12.4 and 6.8%, respectively) in comparison with the first cycle of production (Fig. 1). In the two years of research the yield of plants grown in re-used rockwool exposed to phytosanitary treatments was higher than in control variants. In autumn growing in two and three-year-old control slabs, there was a distinct decrease of the yield in relation to the yield obtained in the first year of using rockwool, especially in 2001 (Fig. 1). The phytosanitary treatments prevented to a different extent autumn tomato yield from decreasing in consecutive cycles of growing in the same rockwool. In this case in the two consecutive years, chemical disinfection of slabs integrated with the use of *P. oligandrum* proved to be the most effective. Additional applications of *P. oligandrum*, *T. viride* B35 and the fungicide mixture increased the marketable yield in comparison with the disinfectant alone, on the average, by 3.2, 4.0 and 4.9% in the spring crops and by 11.8, 2.1 and 6.9% in the autumn crops, respectively.

In autumn crop of the year 2001, regardless of the growing cycle, the average level of root rot severity in treatments involving introduction of *T. viride* B35 and application of fungicides was significantly lower in comparison with the control. All the compared treatments of rockwool slabs significantly reduced the severity of symptoms of the basal stem and crown rot, caused by *Pnn*. In the year 2002 in autumn growing, similarly to spring cycle, none of the compared treatments of rockwool slabs caused significant improvement of the root health status (data not shown). Although *Forl* was present in the substrate, no apparent aboveground disease symptoms due to this pathogen were observed.

The results obtained indicate that growing greenhouse tomatoes for three consecutive years in the same rockwool slabs does not pose the risk of a significant decrease of the marketable yield, provided that chemical or integrated disinfection of growing substrate between successive crops is conducted. Although only in one out of four trials chemical disinfection of re-used rockwool slabs integrated with a complementary introduction of a biocontrol agent resulted in a significant increase of the fruit yield compared to the QAC+Chx disinfectant used alone. Integration of chemical and biological methods can be considered as a potential phytosanitary measure enabling to extend the use of rockwool slabs over a period of three years. In this context, the present study can be regarded as a contribution to Katan’s recent statement postulating, “Integrating biocontrol with disinfection is a very promising approach for research which deserves to be further developed and intensified” (Katan, 2000).

**References**

Risk assessment
Can biotechnology help biocontrol to overcome its innate weaknesses?

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Abstract: Genetic manipulation of microorganisms with the goal of increasing biocontrol abilities by introducing additional mechanisms is feasible and several articles are published. Increased potential risks of such genetic modified microorganisms are also recognized and possible safeguard mechanisms discussed. In this paper a short up to date discussion is presented. Currently risk assessment seems to be not able to answer any of the relevant safety questions, therefore it seems too early to envisage any practical use of biotechnology to overcome biocontrol limitations.

Key words: biocontrol agent, transgenic, risk assessment

Biocontrol agents (BCAs) are praised as potential alternatives to the much questioned synthetic pesticides. The most successful biocontrol agents are microorganisms, mites or insects which control pests imported into a foreign environment by humans. Several examples can be found under the flag “mycoherbicide”, which mainly consist of the control of a noxious weed, often imported long ago, by introducing a pathogen able to keep the weed under control in its centre of origin. These approaches can be defined as “classical biocontrol”. In plant pathology similar examples can be also found, i.e. the reduction of Cryphonectria parassitica aggressiveness on chestnut in Europe by hypo-virulence associated with infections by fungal viruses (family Hypoviridae), which most probably originate in the same area of the fungus and has been introduced in Europe after it. This type of biocontrol is nothing else than a re-establishment of the “natural equilibrium” where potential high reproduction rates of a particular organisms are counteracted by its enemies. However, cases where classical biocontrol can be applied are rare; in most cases we have to operate in situation where the target pest and its biocontrol agent are subject to co-evolution. In this paper we analyse the latter type of biocontrol agent. Why is there such an interest in biological control? Even if the answers may be highly differentiated, finally it boils down to potential risk and fear of the abuse and misuse of synthetic pesticides.

Biocontrol agents, contrary to synthetic pesticides, are reputed to have no negative impact on the environment nor are suspected to leave unwanted residues on food, often without any confirming data. The argumentation is that being them already present in nature, negative environmental effects or human health endanger would have already been recognized. For example, if the organism was invasive and toxic to flora and fauna it would have displaced its opponents during evolution. These particular organisms must rather be rare as scientists had to devote much time and resources to find it. There is some truth to this argumentation: evolution will eliminate organisms which are “too successful” in parasitizing and favour organisms which allow survival of their hosts. This argumentation is reassuring from the environmental point of view. If we consider these arguments from the viewpoint of biocontrol, we may conclude that a biocontrol organism as we desire (rapidly killing or
eliminating, even at extremely low presence, the target), does not exist, as these abilities would be highly disadvantageous from the evolutionary point of view.

Is therefore the idea of using living organisms to control pest and diseases doomed or at least relegated to a niche such as organic agriculture? With the development of the DNA-recombinant technology, genes from other organisms can be introduced. In bacteria the introduction is extremely easy and even the much debated problems, as insertion sites or selection genes, are solved. Clearly it is tempting to manipulate a natural biocontrol organism from a lazy frustratingly inefficient status to a sparkling combat ready organism. This “genetic doping” opens complete new perspectives. Several biocontrol organisms have been manipulated already and a long list of publications is available, much less is published on the application and testing of genetically modified (GM) microorganisms in field conditions. An immediate reaction of general public policy makers and scientists, at least in Europe, would be to question the legality of any field trial. As much as working with GM-bacteria in laboratories is unquestioned, any open environment testing of GM-bacteria is strictly regulated in Europe.

Bacteria are organisms which are out of our control once set free; we can not follow the fate of a single bacterium once it is out of a confined area. Unwanted plants can in most cases be hindered from uncontrolled expansion, GM-plants can be confined in experimental set ups until proof of absence or acceptable risk is established. GM-bacteria peped up to be more aggressive, to exert a more lethal action or to persist better are beyond our control.

We will illustrate a few cases of peped up biocontrol agents created in various labs and launch the discussion if such “GM-biocontrol agents” should be used and, in the case of affirmative response, what would be the minimal standard of risk assessment. Baiton et al. (2004) complemented a good root colonizing strain of *Pseudomonas fluorescens* with the antibiotic 2,4-diacetylphloroglucinol gene. As authors state, the new strain may possess the necessary qualities for effective biocontrol. They observed also a lowered competence of the strain in the surrounding soil, deducing that this can lower the risk of uncontrolled spread.

Timms-Wilson et al. (2004) were able to pep up a *P. fluorescens* strain so that it produced phenazine-1-carboxylic acid. The transgenic strain showed a good suppression of *Pythium* even at high densities and also had an enlarged spectrum of activity. They evaluated the impact of the strain on the soil microbial community and on mycorrhizal associations and found only transitory and minor perturbations, coming to the conclusion that the plant species, development stage and diseases had greater impact on the rhizosphere diversity than the introduction of the wild type or the transgenic biocontrol organisms. Gerhardson (2002) views the possibility, if GMOs could be used unrestricted, to design new organisms with improved efficacy and activity spectrum, by enhancing expression of relevant genes and combing genes from different biocontrol agents.

Companies especially in the 1990th have been awarded patents. In the last years large companies seem to have mostly abandoned BCA and GM-BCAs, however some smaller ones, like Bioworks Inc., deposited patents on genetically modified BC-strains for use in plant disease control and recombinant bioactive molecule delivery. An example is the patent “New transgenic *Trichoderma* species comprises a recombinant nucleic acid molecule encoding a bioactive molecule, useful for controlling plant diseases and delivering bioactive molecules to plants and plant seeds”. Quite a few examples can be found in the scientific literature all dealing with research and development of GM-BCAs in controlled environment.

Field trials with GM-BCAs are, to our knowledge, neither reported nor authorized in Europe. Parallel with concretization of the idea to boost biocontrol activity through DNA engineering, publications appear voicing concerns (Spadaro & Gullino, 2005) and proposing possible ways to avoid unwanted effects (failsafe mechanisms). The most relevant safety
issues concern uncontrolled spread, displacement of non target microorganisms, allergenicity, toxicity, pathogenicity to non target organisms, genetic and transgene stability. The mechanisms proposed (Gressel, 2001) to prevent or at least mitigate uncontrolled spread of biocontrol microorganism or transmission to other organisms of the transgenes from transgenic microorganism rendered hyperaggressive, touch the reproduction and spore germination ability and the formation of viable spores, appressoria, spore stalks and melanin. However the proof of the validity of concept has not been made and it is probably impossible to do so.

Currently risk assessment seems not to be able to answer any of the relevant safety questions, therefore it seems too early to envisage any practical use of biotechnology to overcome biocontrol limitations.

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Development of risk assessment methodology for biocontrol agents

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Abstract: Regulatory guidance on the risk assessment of Biocontrol Agents (BCAs) needs a strong impulse as risk assessors need to evaluate 15 ‘existing’ BCAs. Today, regulatory guidance is still limited. A risk decision tree was developed which guides risk assessors and regulators through the risk assessment. The decision tree is primarily based on the data requirements (Annex IIB and IIIB) and the uniform principles (Annex VIB) of 91/414/EEC.

Keywords: decision tree, methodology, regulatory guidance

Introduction

Micro-organisms used for crop protection are relatively safe when applied as prescribed. However, they always require a proper pre-market safety evaluation in view of potential toxicity, infectivity and pathogenicity. This is of particular importance as plant protection products with active micro-organisms will be increasingly important for crop protection worldwide, primarily due to their allegedly less hazardous impact on human health and the environment when compared with synthetic chemical pesticides. The regulatory 'hurdle', however, is often felt to be one of the most burdensome. Regulatory guidance is limited in this respect. Therefore we developed a risk decision tree (see figure) in cooperation with the National Board for the Authorisation of Pesticides and the Dutch Ministry of Environment. Immediate causes for the proposal were: a) the safety evaluation by the European Union of 15 'existing' BCAs in 2005/2006, and b) harmonisation efforts by OECD/BPSG on safety evaluation of BCAs. The decision tree is currently used as a draft guidance document (Mensink, 2005).

Material and methods

A decision tree has been used as a format for regulatory guidance. Risk descriptions criteria as embedded by the EU were the premises for this tree (uniform principles Annex VIB). However, in view of harmonisation, Organisation for Economical Cooperation and Development and the BioPesticide Steering Group which is an OECD initiative (OECD/BPSG) and North American Free Trade Association (NAFTA) descriptions and procedures have been taken into account when possible.
Figure 1. Environmental risk decision tree for microbial plant protection products (proposal for first tier evaluation). PEC: predicted environmental concentration; EC_{50}: the median effective concentration; NOEC: no-observed-effect concentration; TOs: target organisms; NTOs: non-target organisms; GAP: good agricultural practice.
Results

The decision tree refers mainly to the first tier safety evaluation, i.e. based on rather conservative assumptions as direct and worst case exposure, whereas the actual exposure may be much less or indirect. Fig. 1 presents the importance of thorough information on characterisation, identification and efficacy (box 1 of Fig. 1). The tree also indicates that sufficient and valid data should be available on the emissions (box 2), exposure (box 3) and effects (box 4) on non-target organisms. The tree first focuses on potential toxic effects of a microbial product (sub 4A). If the microbial product is toxic, the safety evaluation proceeds as the EU evaluation of a chemical synthetic pesticide (sub 5C, 5D). Only if a microbial product is not toxic, allergenic or competitive to a non-target organism (NTO), will the tree zoom in on the potential infectivity and pathogenicity. The application rates in tests are preferably those in accordance with Good Agricultural Practice (GAP) (sub 4A,4B).

Discussion

An environmental safety evaluation without data is impossible. However, depending on the submitted data and the overall picture that emerges, some data can be waived (sub 5A). Bridging and familiarity studies to support extrapolation of pathogen strain or type A to strain or type B may be helpful in this respect (OECD, 2006). Taxonomy based on molecular, biochemical or genetic characteristics may be helpful as well.

In comparison with the EU and NAFTA approach, the decision tree leaves more room for expert judgement and discussion in some aspects. The EU, for instance, states that when the exposure of NTOs or compartments is negligible, the risk is always acceptable (from 3A directly to 6B), whereas the proposed decision tree has build in the possibility of scientific or regulatory reflection (via 3D). In the first tier evaluation the NAFTA approach, for instance, - coming from box 2 - focuses directly on box 4, taking box 3 into account not earlier than in the second tier evaluation. The decision tree is more conservative, as it proposes a first tier exposure assessment via box 3.

The necessity of the evaluation of the exposure in the first tier might be reconsidered. A large volume of published work on the persistence of BCAs in the soil indicates that concentrations can be sustained for a certain period of time. It is evident that the BCA should be present at high levels for a certain period of time in order to control the pest organism. In time, a decrease to normal levels is always observed (unpublished results). This work should be taken into consideration in order to serve as a general statement. This work also interacts with REBECA, an EU policy support action. REBECA reviews possible risks of BCAs, compares regulation in the EU and the USA and proposes alternative, less bureaucratic and more efficient regulation procedures maintaining the same level of safety for human health and the environment but accelerating market access and lowering registration costs (REBECA, 2006).

Conclusions

A risk decision tree is proposed as a registration tool for BCAs prior to their marketing. This tree and the accompanying guidance document (Mensink, 2005) should enable risk assessors and environmental scientists to verify the risk criteria, with respect to the potential environmental behaviour, fate and effects of a BCA under review. In this way, it should be possible to discern whether a risk is acceptable or not. Case by case expert judgement, however, remains necessary in cases of limited knowledge of modes of action, population
dynamics and microbial ecology, limited experience with regulatory test protocols, taxonomical difficulties in relation to the indigenousness of active micro-organisms and difficulties in extrapolating laboratory data to the field or short-term effects to a longer term (Hokkanen & Hajek, 2003; Mensink & Scheepmaker, 2007). Long-term effects of BCAs are also not well studied. Uncertainties in the environmental safety evaluation of BCAs need to be counterbalanced in the short term with appropriate guidance documents or working documents. Also general statements, such as one prepared on persistence of BCAs in the soil, help to reduce the data requirements.

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REBECA 2006: http://www.rebeca-net.de
Environmental fate of the biocontrol agent of fire blight

*Pseudomonas fluorescens* EPS62e on apple and pear using real-time PCR and selective media

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**Abstract:** The strain EPS62e of *Pseudomonas fluorescens* was isolated from a healthy pear fruit and is a reliable biological control agent of fire blight because of its high efficacy in controlling *Erwinia amylovora* infections in immature fruits, blossoms and shoots. To develop this biocontrol agent as a commercial biopesticide, the knowledge of its environmental fate after field release is needed. In the present work, the dynamics of EPS62e population on apple and pear blossoms, fruits and leaves was monitored under greenhouse and field conditions. Two monitoring tools were developed and used simultaneously to evaluate the behaviour of the target strain; a method based on CFU counting on selective media, and a real-time PCR method (Pujol et al., 2006a) based on a SCAR marker (Pujol et al., 2005). The biocontrol agent showed an active colonisation of flowers under greenhouse and field conditions, reaching population levels from $10^7$ to $10^8$ CFU/blossom at the carrying capacity of flowers. Strain EPS62e almost dominated completely the cultivable microbiota of flowers. The field trials, carried for about two months, showed that the vast majority of the biocontrol agent population remained at the calyx area after the fruit set. In blossom trials, no significant differences were observed between population levels assessed by CFU-counting and real-time PCR methods. However, when EPS62e was inoculated in leaves, the population levels decreased during time, and the values estimated by both methods significantly differed under greenhouse conditions. In general in leaves, values obtained by real-time PCR were higher than those obtained by CFU-counting, indicating a possible entry into a viable but unculturable (VBNC) state of a part of EPS62e population and the presence of non-degraded DNA after cell death. However, under field conditions, the population levels on leaves decreased till non-detectable levels and both methods of analysis coincided. The results indicated that the biocontrol agent was under stressful conditions when inoculated in the phyllosphere and may enter into a VBNC state or dead, whereas it was under optimal colonisation conditions when applied in blossoms. Therefore, the use of both monitoring methods provides useful information on epiphytic fitness of the biocontrol agent depending on the host species, the plant organ and weather conditions.

**Keywords:** monitoring

**Introduction**

Fire blight is a serious disease caused by *Erwinia amylovora* that affects several plant species, mainly belonging to the rosaceous family. The disease is worldwide distributed, and causes severe economical losses in crop production (Vanneste, 2000). Traditionally, fire blight management has been based on chemical control strategies; however, biological control is increasingly used in some countries (Stockwell et al., 2002). Biological control of fire blight is based on the establishment of the biocontrol agent in blossoms prior to the pathogen arrival. The epiphytic growth of the biocontrol agent is determined by the physical and
microbiological environment. Therefore, the study of the epiphytic fitness of the biocontrol agent under different conditions is crucial to assure its efficacy.

*Pseudomonas fluorescens* EPS62e was isolated from a healthy pear fruit in Girona (Spain) and it was selected for its high efficacy in controlling *E. amylovora* infections (Pujol et al., 2005). In order to evaluate its environmental fate after field release, two monitoring methods were developed and validated: a molecular method based on a strain-specific real-time PCR, and a cultivable method based on CFU-counting on selective media (Pujol et al., 2005; Pujol et al., 2006a). In the present work, the combined use of molecular and cultivable monitoring methods was used to evaluate the epiphytic fitness of *P. fluorescens* EPS62e after its application on apple blossoms and leaves, and on pear blossoms under greenhouse and field conditions. We also evaluated the impact of EPS62e on the indigenous bacteria, and its ability to spread from treated to non-treated trees within the orchard.

**Materials and methods**

**Bacterial strains, growth media and DNA extraction**
A spontaneous nalidixic acid-resistant mutant of *P. fluorescens* EPS62e was obtained in the laboratory. For routinely use, the strain was cultured in Luria-Bertrani agar (LB) supplemented with 50 mg L\(^{-1}\) of nalidixic acid at 25ºC for 24 h. For each monitoring assay, independent inoculum suspensions were prepared in sterile water and adjusted spectrophoto metrically at 10\(^8\) CFU mL\(^{-1}\). For real-time PCR, DNA was extracted as described by Llop et al. (1999) with minor modifications (Pujol et al., 2006a).

**Greenhouse and field trials**
Monitoring assays were performed in blossoms and leaves of the Golden Delicious apple cultivar and blossoms of the Doyenne du Comice pear cultivar. Greenhouse trials were only performed with apple blossoms and leaves, whereas field trials were performed with apple trees during spring-summer 2004 in Angers (France), and with apple and pear trees during spring-summer 2005 in Girona (Spain). Trials consisted of a single treatment with EPS62e at 10\(^8\) CFU mL\(^{-1}\) until the runoff point. Under greenhouse conditions, detached apple branches were forced to bloom for blossom trials and seedlings were used for leaf trials. Under field conditions, apple and pear trees were treated during full bloom for blossom trials and two months later for leaf trials. To evaluate the spread of EPS62e within the orchard, non-treated trees situated at 15 to 30 m far from the inoculation site were periodically sampled.

**Assessment of *P. fluorescens* EPS62e population level and total cultivable bacteria**
In all trials performed, the first sampling time was carried 12 h after inoculation. Three samples were collected from each trial at each sampling time. Then, samples were periodically taken. Flowers were sampled by bulking six blossoms during 7 days in the greenhouse trials and during 54 days in the field trials. Leaves were periodically sampled by bulking 6 leaves of the greenhouse trials and 12 leaves of the field trials. Samples were transported to the laboratory and homogenized as described by Pujol et al. (2006a). Each sample was analysed twice by CFU-counting methods and real-time PCR methods.

For CFU-counting method, each sample was serially diluted when needed and dropped in triplicates on LB agar supplemented with 50 mg L\(^{-1}\) of nalidixic acid and 50 mg L\(^{-1}\) of econazole salt for EPS62e population measurement and on LB agar supplemented with 50 mg L\(^{-1}\) of econazole salt for total cultivable bacterial population measurement. Colonies were counted after incubation at 25ºC for 48 h.

For real-time PCR, 1 mL of the sample followed the DNA extraction protocol, and each sample was analysed in triplicate by using the TaqMan probe 450 PBR and primers Q 450F and SCAR 450R as described by Pujol et al. (2006a).
Results

EPS62e colonised efficiently blossoms whatever the species (pear and apple) and the climatic conditions. Under greenhouse conditions, EPS62e reached $10^7$ CFU blossom$^{-1}$ at the first sampling time and it remained rather stable till the end of the assay. Under field conditions, the population level of EPS62e started at around $10^6$–$10^7$ CFU blossom$^{-1}$ and stabilized at $10^7$ CFU organ$^{-1}$ under Mediterranean climatic conditions or increased to $10^8$ CFU organ$^{-1}$ under Atlantic climatic conditions, and remaining stable till the end of the assay. At the last sampling time, we observed that up to 99% of EPS62e population was restricted to the calyx area of the immature fruits. In blossoms, there were not significant differences between values obtained by real-time PCR and CFU-counting methods, except at the first sampling time, where in almost all trials real-time PCR values were about two orders of magnitude higher than those values assessed by CFU-counting.

In leaf trials EPS62e poorly survived or dead. Under greenhouse conditions, cultivable population levels decreased with time from $5 \times 10^7$ to $10^4$ CFU g (fresh weight)$^{-1}$, whereas the population level assessed by real-time PCR started at the same value but only decreased to $10^6$ CFU g (fresh weight)$^{-1}$. This difference of two orders of magnitude between methods was maintained from the day 7 to the end of the assay. In field trials under Atlantic climatic conditions, the population assessed by real-time PCR and CFU-counting methods in leaves differed significantly. Real-time PCR values showed a progressive decrease of EPS62e population from $10^7$ to $5 \times 10^3$ CFU g (fresh weight)$^{-1}$ at the end of the assay, whereas cultivable population levels remained low and stable during the assay at about $5 \times 10^3$ CFU (fresh weight)$^{-1}$. A different situation was observed under Mediterranean climatic conditions, where both methods of analysis coincided in the population assessment except at the first sampling time. In this case, EPS62e population level decreased with time till undetectable levels 30 days after treatment.

EPS62e accounted for 100% of the total cultivable bacterial population in inoculated apple and pear blossoms under field conditions from few days after treatment to the end of the assay. Contrarily, in apple leaves under field conditions, EPS62e was not able to dominate the habitat since total cultivable bacterial population remained stable between $10^4$ and $10^5$ CFU g (fresh weight)$^{-1}$ and EPS62e population decreased with time.

The biocontrol agent EPS62e was detected in all non treated apple and pear blossoms situated at 15 to 30 m far from the inoculation site. In general, the population started $10^3$ CFU blossom$^{-1}$ and increased significantly to $10^5$–$10^6$ CFU blossom$^{-1}$ 7 days later, although it was a high variability between trials. Very low values of EPS62e population were sporadically observed in apple leaves.

Discussion

*Pseudomonas fluorescens* EPS62e has shown to be an efficient coloniser of blossoms of different plant host species (apple and pear trees) under different weather conditions (greenhouse and field under Atlantic and Mediterranean climatic conditions), compared to other biocontrol agents of fire blight such as *Pantoea agglomerans* C9-1 (Nuclo et al., 1998) and *P. agglomerans* Eh252 (Johnson et al., 2000).

The phyllosphere is less appropriate than blossoms for EPS62e colonisation, since in all trials performed it only remained at very low levels or even dead. Probably, the harsh environment of leaves with low availability of nutrients (Lindow & Brandl, 2003) ad the summer weather conditions prevents EPS62e multiplication.
The simultaneous use of two monitoring methods, CFU-counting and real-time PCR, provided the identification of three physiological states for EPS62e in the aerial part of plants which consisted of (i) an active colonisation of blossoms, where both methods gave similar and increasing values; (ii) the entry into a VBNC state on leaves under suboptimal conditions, where population initially decreased and stabilized after with values of real-time PCR two orders of magnitude higher than those obtained by CFU-counting; and (iii) death, where population levels progressively decreased to undetectable levels according to both methods of analysis (Pujol et al., 2006b).

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Development of a quantitative competitive PCR assay for the quantification of the biocontrol agent *Pseudomonas fluorescens* Pf153 in soil

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Abstract: *Pseudomonas fluorescens* Pf153 was isolated from the roots of tobacco plants grown in a soil suppressive to black root rot caused by *Thielaviopsis basicola*. We developed two PCR-based molecular markers (Pf153_1 and Pf153_2) that allow the specific identification of the strain Pf153 among pseudomonads and other bacteria present in the soil. Based on the marker Pf153_2 a quantitative competitive PCR assay was developed. The competitor cmp2 was cloned into the plasmid pME6031 and transformed into *P. fluorescens* CHA0. In soil the DNA extraction efficiency from both *Pseudomonas* strains is identical; therefore CHA0/c2 can be used as a standard to quantify the biocontrol strain Pf153. The detection limit of QC-PCR lies between 6.6 and 0.66 Pf153 CFU/mg soil. This method enables an exact quantification of Pf153 in biocontrol assays performed in natural soil, overcoming differences in DNA extraction efficiency and PCR amplification from any soil environment.

Keywords: DNA extraction internal control, monitoring, risk assessment

Introduction

Among the many organisms suited for biocontrol of soil borne diseases, the root-colonizing bacterium *Pseudomonas fluorescens* Pf153 is a valuable candidate (Fuchs & Défago, 1991). It was isolated from the roots of tobacco grown in Morens soil (Switzerland), which is suppressive to *Thielaviopsis basicola* (Fuchs et al., 2000).

In order to perform risk assessment studies with Pf153 as a biocontrol agent, following three objectives were fixed (1) the design of at least one reliable genetic marker allowing discrimination of Pf153 from soil microorganisms, (2) the development of a quantitative-competitive PCR for the quantification of Pf153 in soil using a single PCR marker, and (3) the establishment of a soil DNA extraction methodology able to overcome differences in DNA extraction yields from different soils. These goals were achieved thanks to a genetically engineered reference *Pseudomonas* strain containing the competitor for QC-PCR added to each sample in the first phase of soil DNA extraction.

Materials and methods

**Pf153-specific markers and bacterial standard**
Forty six bacterial strains from different geographic regions worldwide were selected. RAPD-PCR amplifications of cell lysates were carried out to recover genetic diversities among the
bacterial strains. Putative Pf153-specific bands were selected, excised and sequenced. Specific primers were designed.

The competitor oligo-nucleotide cmp2 was constructed by PCR as described by Celi et al. (1993) using Pf153_2_rev and Pf153_2_cmp primers for SCAR Pf153_2. The competitor cmp2 was cloned into the vector pME6031. Electro-competent P. fluorescens CHA0 bacteria were transformed with the resulting plasmid, creating P. fluorescens CHA0/pME6031-cmp2 (CHA0/c2).

**CFU determination for Pf153 and CHA0/c2 and calibration of the QC-PCR**

Bacteria were grown in LB until an OD$_{600}=0.125$. Serial dilutions were performed in saline (0.9% NaCl) and 40 µl of the $10^{-5}$ dilution were plated in triplicate on LB plates. To determine the ratio OD$_{600}$(Pf153)/OD$_{600}$(CHA0/c2) at which the QC-PCR generates amplicons of the same intensity, 10 µl of Pf153 and 10 µl of CHA0/c2 LB bacterial cultures (OD$_{600}=0.125$) were diluted $10^3$ and $2 \times 10^3$ times in water respectively. A QC-PCR was performed in 40 µl volume using between 1 and 16 µl of the CHA0/c2 cell lysate and 4 µl of the Pf153 cell lysate.

**Establishment and validation of Pf153 quantification in soil**

Four “E tubes” included in the FastDNA SPIN Kit for Soil were filled with 100 mg peat (T) each. Bacterial strains Pf153 and CHA0/c2 were grown until an OD$_{600}=0.043$ and 0.433, respectively. Fifty microliters of the Pf153 culture were inoculated in the first “E tube”. This inoculated soil represents an unknown soil sample (USS) from which Pf153 should be quantified. The reference soil (RefS, contained in the second E tube) was inoculated with 50 µl of the CHA0/c2 bacterial culture. The remaining two peat containing “E tubes”, used as a lysis and extraction controls, were inoculated with both 50 µl Pf153 liquid culture and with 50, 5 µl (SC1, third E tube) or 5 µl (SC2, fourth E tube) CHA0/c2 liquid culture. The four soils (USS, RefS, SC1 and SC2) were incubated for 3 h at room temperature. To the USS and RefS soils, 978 ml of SP- and 122 ml of MT buffer were added. The bacteria were lysed by homogenization for 30s at a speed of 5.5 in the homogenizer and the resulting mixture was centrifuged. From the 600 µl USS supernatant, four aliquots of 130 µl were transferred to four new 1.5 ml EP, each. The supernatant of the RefS sample (RefS1) was diluted 10 (RefS2), 100 (RefS3) and 1000 times (RefS4). The solutions RefS1, RefS2, RefS3 and RefS4 (130 µl each) were added to the 130 µl of the four Pf153-containing aliquots, originating the samples USS1, USS2, USS3 and USS4, respectively. From this step on, the protocol was followed as described by the manufacturer. The soil control samples SC1 and SC2 were processed using the exact protocol as described by Qbiogene. In parallel, two bacterial culture mixes were prepared both with 50 µl Pf153 liquid culture (OD$_{600}=0.043$) and with 50 µl (CP1) and 5 µl (CP2) CHA0/c2 liquid culture (OD$_{600}=0.433$). The ratio between the amount of the bacterial strains in PC1 and in SC1 is identical. The same is valid for the PC2 and the SC2 samples.

**Results**

**Pf153 diagnostic PCR**

The primer F6 gave rise to two Pf153-specific fragments. Specific primers were designed (Table 1). In diagnostic PCR, primers pf153_1 and pf153_2, generated the corresponding SCAR fragment from genomic DNA of P. fluorescens Pf153. A multiplex PCR assay on the 46 bacterial strains showed that the markers Pf153_1 and Pf153_2 unequivocally distinguish P. fluorescens Pf153 from closely related fluorescent pseudomonads and from other bacteria.
Table 1: 

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>Eas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf153_1</td>
<td>Pf153_1_for:</td>
<td>CAAGCACCGTTGCAATTAGA</td>
<td>for/rev: 162 bp</td>
</tr>
<tr>
<td></td>
<td>Pf153_1_rev:</td>
<td>ACATGACACTGCTGGGTTTG</td>
<td></td>
</tr>
<tr>
<td>Pf153_2</td>
<td>Pf153_2_for:</td>
<td>GCATCAGACTCTCCCGATTG</td>
<td>for/rev: 261 bp</td>
</tr>
<tr>
<td></td>
<td>Pf153_2_rev:</td>
<td>GACGTTGGGACGGGTATTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pf153_2_cmp:</td>
<td>CTCCCGAAGCTTCTTTCAGG</td>
<td>cmp/rev: 208 bp</td>
</tr>
</tbody>
</table>

Optimal primer annealing temperatures are 60°C. Eas: expected amplicon size.

**CFU determination and QC-PCR calibration**
On average $9.9 \times 10^7$ (sd: 1.4) CFU Pf153 and $1.75 \times 10^7$ (sd: 0.5) CHA0/c2 CFU were contained in one millilitre LB liquid culture. When the bacteria are mixed in a ratio $\frac{OD_{600(Pf153)}}{OD_{600(CHA0/c2)}} = 1.06$ the QC-PCR generates amplicons of the same intensity.

**Validation and of Pf153 quantification in soil**
The ratio between the Pf153 DNA and the CHA0/c2 DNA was conserved in the USS2 eluate, SSC2 eluate and PC2 cell culture lysate. The number of Pf153 CFU present in the original USS can be calculated using the experimental values related to the reference strain CHA0/c2 for the QC-PCR closer to the equilibrium (Fig. 1, lane USS2): the $OD_{600}$ (CHA0/c2) must be multiplied for the dilution factor of the soil lysate, the volume of CHA0/c2 liquid culture pipetted in the RefS, the constant $c$ ($1.75 \times 10^7 \times 5.32 / 0.125 = 7.45 \times 10^8$ CFU ml$^{-1}$) and divided by the band intensity ratio. According to the formula, $1.65 \times 10^6$ CFU of Pf153 were inoculated (3.2% underestimation of the amount really inoculated in the USS; $1.7 \times 10^6$ CFU). The USS3 and USS4 samples showed a Pf153-derived amplicon of increasing intensity as the quantity of the reference strain diminished.

**Discussion**
The method reported delivers a sensitive, reliable and cost-effective monitoring method that can be properly applied to estimate the spread and colonization of the biocontrol agent Pf153 in soil during biocontrol and risk assessment studies. It features two main benefits that are rarely combined in any microorganism quantification procedure: (1) it is unaffected by the soil DNA extraction efficiency and (2) every soil sample includes a positive QC-PCR control. The addition of CHA0/c2 to every soil sample acts as an extraction internal standard: as the lysis- and DNA extraction efficiencies are identical for both bacteria, the ratio between their DNAs in the elute, after extraction, reflects the DNA ratio of the bacteria in the soil. The addition of the reference strain therefore enables the correct quantification of Pf153 independently of soil characteristics.

CHA0/c2 also acts as an internal control in every QC-PCR reaction. As this strain is present in every sample, a QC-PCR producing weak signals or no amplicon indicates that the DNA extraction procedure may not have been successful or that polymerase-inhibitors may be present in the eluate. The QC-PCR presented in this study quantifies the amount of Pf153 DNA extracted from soils with a minimum sensitivity included between $6.6 \times 10^3$ and $6.6 \times 10^5$ CFU g$^{-1}$ soil. This limit of detection is lower than the minimum population density required for significant disease suppression. (Haas et al., 2000). In the future this procedure can easily
be adapted for the detection of any other Pseudomonas strain just by replacing the competitor used here, with one derived from another SCAR marker specific for the strain of choice.

Figure 1. Validation of Pf153 quantification in an unknown soil sample (USS) after QC-PCR. Four equal Pf153-containing soil lysate aliquots were mixed with four tenfold dilutions (10^0, 10^-1, 10^-2 or 10^-3) of a soil lysate containing the reference strain CHA0/c2 (USS1; USS2; USS3; USS4, respectively). In soil controls SC1 and SC2, Pf153 and CHA0/c2 were co-inoculated in soil in different ratios prior to DNA extraction. In PCR controls PC1 and PC2, Pf153 and CHA0/c2 cultures were mixed in different ratios and no DNA extraction was performed. M: 100-bp ladder, W: PCR negative control (water).

Acknowledgements

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References

Development of a RAPD marker and a semi-selective medium for *Aureobasidium pullulans* (strain Ach1-1), a biocontrol agent against post-harvest diseases on apples

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Abstract: *Aureobasidium pullulans* strain Ach1-1 is an efficient biocontrol agent against *Botrytis cinerea* and *Penicillium expansum* on apples. Specific tools have to be developed in order to monitor this biocontrol agent. This will contribute to evaluate its adaptation and its survival ability on post-harvest commodities or to assess the effectiveness of various application methods. The monitoring of the antagonist has been achieved following two complementary approaches. On the one hand, a specific RAPD marker (522 pb) to strain Ach1-1 has been obtained among a collection of 11 strains of *A. pullulans* with primer OPR-13. This fragment was cloned and sequenced in order to develop a SCAR marker for the strain Ach1-1. On the other hand, a semi-selective medium for quantification of strain Ach1-1 is under development. This medium presents a high toxicity towards the air microflora, while the growth of strain Ach1-1 was unaffected.

Keywords: monitoring, SCAR

Introduction

The yeast-like fungus *Aureobasidium pullulans* (De Bary) Arnaud strain Ach1-1, Ach2-1 and Ach2-2 were isolated in the Plant Pathology Unit of F.U.S.A.Gx (Belgium) from *Golden Delicious* for their high antagonistic activity (more than 80%) against *Botrytis cinerea* and *Penicillium expansum*, two major pathogens of post-harvest apples (Achbani et al., 2005). *A. pullulans* strains are currently investigated for their biological control ability on various fruit and vegetable models (Ippolito et al., 2000; Schena et al., 1999). However, among the multiple stages leading to the commercialization of a biopesticide, the monitoring of the strain constitutes an important step. Monitoring tools are required to specifically identify the studied strain among a complex population of microorganisms. Furthermore, it will allow tracking the population evolution according various application techniques or formulations under different environmental conditions. Several methods were developed to follow population dynamic of biocontrol agents. A technique combining dilution plating on a semi-selective medium and strain-specific identification with a SCAR (Sequence Characterized Amplified Region) marker was developed by De Clercq et al. (2003). This technique was recently improved by the use of real time PCR, allowing the combination of SCAR and Taqman fluorescent chemistry (Massart et al., 2004).

In this study, RAPD (Random Amplified Polymorphic DNA) was used for identification of a specific molecular marker for *A. pullulans* strain Ach1-1. This specific fragment was
sequenced in order to obtain a SCAR marker specific to the strain Ach1-1. A semi-selective medium for the strain Ach1-1 was also developed.

**Material and methods**

**Aureobasidium strains and culture conditions**

*A. pullulans* strains used in this study are described in Table 1. Before each experiment, the strains were grown on potato Dextrose Agar (PDA) (Merck, Germany) at 25°C during 48 h, for three successive generations. For DNA extraction, strains were inoculated into YEPD (yeast extract 1%, peptone 2%, dextrose 2%; Merck) at 25°C over 24 h with agitation.

Table 1. Origin and source of *A. pullulans* strains

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Strains</th>
<th>Original Substrate</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. pullulans</em></td>
<td>Ach1-1</td>
<td>apple, Golden Delicious (Belgium)</td>
<td>Institut National de Recherches Agronomiques-Meknès (Morocco)</td>
</tr>
<tr>
<td></td>
<td>Ach2-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ach2-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MUCL 22377</td>
<td>Leaves; <em>Quercus robur</em> (Sweden)</td>
<td>Mycothèque de l'Université</td>
</tr>
<tr>
<td></td>
<td>MUCL 20326</td>
<td>Leaves; <em>Hordeum sativum</em> (Belgium)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MUCL 20322</td>
<td>tiles (The Netherlands)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MUCL 6147</td>
<td><em>Picea abies</em> (Norway)</td>
<td></td>
</tr>
<tr>
<td><em>A. pullulans</em> var. <em>pullulans</em></td>
<td>MUCL 7862</td>
<td>Cupule; <em>Fagus sylvatica</em> (Belgium)</td>
<td>Catholique de Louvain-La-Neuve (Belgium)</td>
</tr>
<tr>
<td><em>A. pullulans</em> var. <em>melanogenum</em></td>
<td>MUCL 8724</td>
<td>soil (Zaire)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MUCL 19714</td>
<td>fruit; <em>Symphoricarpos rivularis</em> (Sweden)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MUCL 19360</td>
<td>tree; <em>Xylotherus lineatus</em> (Belgium)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MUCL 43163</td>
<td>Leaves and stem; <em>Citrus sinensis</em> (Brazil)</td>
<td></td>
</tr>
</tbody>
</table>

**DNA extraction and RAPD analysis**

*A. pullulans* genomic DNA was isolated according to Ausubel et al. (1987).

The RAPD primers used in this study were obtained from Operon Technologies Inc. (Alameda, California). Thirty nine random primers were tested on extracted DNA of 12 *A. pullulans* strains including strain Ach1-1. The PCR reactions were performed in 25 µl mixture containing 1.0 U of Taq DNA polymerase (Sigma-Aldrich, Inc., USA), 2.5 µl of 10× PCR buffer Sigma, 100 µM of each dNTP, 2.5 µl of MgCl₂, 0.5 µM selected RAPD primer and 50 ng of genomic DNA. PCR amplifications started with an initial denaturation at 94°C for 5 min, followed by 44 cycles at 94°C for 15 s, 36°C for 45 s and 72°C for 90 s with a final extension step at 72°C for 10 min. Aliquots (10 µl) of PCR products were subjected to electrophoresis in 1.5% agarose gel and visualized by staining with 1 µg/ml ethidium bromide.

**Cloning of strain-specific RAPD marker**

The specific RAPD fragment was isolated from the gel with QIAEX II DNA Purification Kit (Qiagen) according to the manufacturer’s instructions. This Fragment was cloned using the TA Cloning kit (Invitrogen, USA). The Ultraprep plasmid DNA (AHN Biotechnologie, Germany) was used to isolate plasmids. The inserts were sequenced in both directions using the BigDye
Terminator 3.1 cycle sequencing Kit (Applied Biosystems). The sequence obtained was analyzed for potential similarity with sequences in GenBank database using BLASTn.

**Semi-selective medium**

Development of a semi-selective medium for the strain Ach1-1 was carried out by various combinations of five fungicides (Sumico, Lirotect, Thiram, Topsin, Euparen), and six antibiotics (Hygromycin B, streptomycin sulfate, spectinomycin, tetracyclin, cycloheximide, Geneticine). Strain Ach1-1 (10^2 cfu) were plated out on each medium (four replicates for each medium) and incubated at 25°C for 7 days. The selectivity of the medium was evaluated against the natural laboratory microflora. Petri dishes were opened during three hours in the laboratory. After one week of incubation at 25°C, colonies were visually observed and enumerated.

**Results**

**Identification of strain-specific RAPD markers**

Thirty nine random primers were first tested with strain Ach1-1 DNA and two other strains Ach2-1 and Ach2-2. Eight primers were selected (OPP-01, OPQ-04, OPQ-12, OPR-06, OPR-13, OPT-01, OPT-05, OP-T17) for their ability to generate at least one specific DNA fragment to the strain Ach1-1. The size of specific bands was ranging between 2.6 and 0.4 kb. Selected primers were then evaluated on the complete *A. pullulans* collection, fragments specific to strain Ach1-1 were obtained with five primers (Table 2).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Number of specific bands</th>
<th>Size of specific bands (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPQ-04</td>
<td>5'-AGTGCGCTGA-3'</td>
<td>1</td>
<td>1060</td>
</tr>
<tr>
<td>OPQ-12</td>
<td>5'-AGTAGGGCAC-3'</td>
<td>1</td>
<td>875</td>
</tr>
<tr>
<td>OPR-13</td>
<td>5'-GGACGACAAG-3'</td>
<td>1</td>
<td>525</td>
</tr>
<tr>
<td>OPT-01</td>
<td>5'-GGGCCACTCA-3'</td>
<td>1</td>
<td>525</td>
</tr>
<tr>
<td>OPT-17</td>
<td>5'-CCAACGTCGT-3'</td>
<td>1</td>
<td>1300</td>
</tr>
</tbody>
</table>

The RAPD patterns obtained with the primer OPR-13 are presented in Fig. 1. Eight to twenty two DNA fragments ranging from 0.4 to 3.2 kb were amplified. The Ach1-1 specific fragment was cloned and sequenced, leading to a 522 bp sequence. Nucleotide homology searches revealed no appreciable similarity with sequences in the GenBank database.

![Figure 1. RAPD patterns of A. pullulans strains obtained with OPR-13 primer. Ach 1-1 (1 and 2); Ach 2-1 (3); Ach 2-2 (4); MUCL 22377 (5); MUCL 20326 (6); MUCL 20322 (7); MUCL 6147 (8); MUCL 8724 (9); MUCL 7862 (10); MUCL 19714 (11); MUCL 19360 (12); MUCL 43163 (13); Negative control (14); M: DNA marker: Generuler™ 100 bp DNA ladder plus (Fermentas); The white arrow indicates the specific fragment.](image-url)
**Semi-selective medium**

Fungicides and antibiotics were combined and the toxicity was evaluated as described in material and methods. The results of some non-toxic combination (% of viability superior to 90%) for the strain Ach 1-1 are represented in Table 3.

Table 3. Analysis of medium toxicity on the growth of *A. pullulans* strain Ach1-1 in comparison to the PDA medium

<table>
<thead>
<tr>
<th>Code</th>
<th>Concentration of fungicides and antibiotics</th>
<th>% of viability ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2</td>
<td>L 10ppm + T 1ppm + SP 25ppm + TR 50ppm</td>
<td>107.8 ±16.76</td>
</tr>
<tr>
<td>S16</td>
<td>S 1.5ppm + T 1.5ppm+ ST 30ppm +G 2.5ppm</td>
<td>101.37±19.24</td>
</tr>
<tr>
<td>S17</td>
<td>S 1.5ppm + T 1.5ppm+ TR 50ppm +G 2.5ppm</td>
<td>102.73±16.36</td>
</tr>
<tr>
<td>S18</td>
<td>E 0.5ppm + S 1ppm+ H 2.5ppm + ST 30ppm</td>
<td>102.74±13.21</td>
</tr>
</tbody>
</table>

Lirotect (L), Thiram (T), Sumico (S), Euparen (E), Hygromycin B (H), streptomycin sulfate (ST), spectinomycin (SP), Tetracyclin (TR), cyclohexymide (C), Geneticine (G)

Selectivity of these combinations was then evaluated on the air microflora. Only the S18 medium showed an important inhibition for the air microflora, allowing the sole development of one small and white filamentous fungus, while PDA medium was at the same time covered with several types of filamentous fungi (Fig. 2).

![Image](image.png)

Figure 2. Toxicity test of the S18 semi-selective medium against strain Ach1-1 and air microflora

**Discussion**

We described here the development of a monitoring technique specifically adapted to strain Ach1-1 of *A. pullulans*, previously isolated for its biological control properties against *P. expansum* and *B. cinerea* combining molecular and microbiological detection tools, our approach will allow an easy quantification of the biological control agent after application but also its accurate identification among various strains of the same species.
Considered as a rapid and simple technique to study genetic polymorphism with population of the same species (Olive & Bean, 1999), RAPD was used here in order to identify a SCAR marker specific to strain Ach1-1.

The specific fragment obtained with the RAPD primer OPR-13 was cloned and sequenced, generating a 522 bp fragment. Starting from this specific sequence, our objective is now to identify a SCAR sequence compatible with real time PCR requirements. Beside the direct quantification of the strain, Real time PCR really improved the rapidity, the sensitivity and the accuracy of the PCR-based detection techniques (Massart et al., 2004).

Our monitoring system also relies on a semi-selective medium, allowing simple identification of the biological control agent by a classical dilution plating method. Despite its lower accuracy, this technique remains an efficient alternative to molecular methods. The medium developed here prevents the growth of air microflora and was not toxic for strain Ach1-1. In further studies, the specificity of our medium will be evaluated against the washing water of apples. This point is of major importance since the semi-selective medium will be used to quantify the antagonist population on apple surface.

**Acknowledgements**

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**References**


Bar code labelling system for managing and tracking microbial culture collections and experiments in labs

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Abstract: Maintaining microbial culture collections, as well as management of safety at work, are relevant aspects in research centres especially in small ones dealing with biocontrol in agriculture. Accurate planning and managing experiments fosters reliability of the scientific data, while saving time and money. LEsyMas is a bar code based mobile information system, developed in order to assist small research centres in carrying out experimental activities in microbiological labs and experimental fields. It is a simple and reliable logging system that allows online mobile gathering of data related to the components of experimental trials with microorganisms (biocontrol agents, plant pathogens, etc.). The bar code labelling also allows managing safety sheets and procedures for using hazardous substances and microorganisms and maintenance of lab facilities. The system is composed of two main components: desktop and mobile (based on a wireless, mobile barcode reader and logger), which are interconnected by a local area network. The system is multilingual, with the possibility to select the preferred language among English, French, German and Italian. The wireless architecture and the handy data-loggers allow portability and reduction of costs with long term benefits in simplifying lab management and in guaranteeing data traceability and reproducibility of trial methodology.

Key words: application, planning and managing experiments, safety management

Introduction

In the forties Woodland and Silver developed the idea of a code (similar to Morse code) useful to save time for cash operations in food industry. They realized an initial bar code capable to automatically identify goods, using photo-multipliers. At the beginning bar code was a “bull’s eye” symbol; a symbol made up of a series of concentric circles. While Woodland and Silver did describe such a symbol, the basic symbology was described as a straight line pattern quite similar to present day bar code. (Woodland & Silver, 1952). The huge size of initial bar code reader, gave some portability problems. Later on thanks to laser technology of scanner and integrated system improvement, bar code gained an amazing success, finding more application (in food industry, in medicine, in public and private utilities). With advances in hi-tech and increasing demand for handheld computing device in economical and commercial fields, more tools for mobile activities (internet and wireless device) have been developed (Hu et al., 2005).

In order to take advantage of the currently available technology we developed this original prototype system called LEyMas (Laboratories Experiment and Management data...
LEXMaS is a logging system using bar code labels that can gather, update or view all information related to three specific elements involved in microbiological and agronomical research activities. The three elements are microorganism (but it is adaptable also macroorganism) collection, chemicals and experiments in labs or experimental fields. This original application was specifically developed for small research institutions and R&D companies dealing with applied microbiology to ensure a quick data storage, access and traceability. The data can be inserted and obtained online thanks to mobile devices (data loggers) and an unequivocal identification system which assign qualitative and quantitative parameters to a specific object in the lab with bar code labels. In this way all data can be available for all users and information can be shared. LEXMaS is a valid tool to identify an object and it can instantaneously provide information on safety and hazardous. Here we present the potential benefits of using a bar code application to manage both experiment data and safety at work.

LEXMaS architecture

LEXMaS system architecture is composed of five components: desktop workstation, mobile device (data-logger), WIFI access point, mobile printer, DB server (Fig. 1). All the information on microorganisms, chemicals and experiments is recorded in a database (DB) (Microsoft SQL Server 2000 Enterprise/Developer Edition).

The desktop workstation gives full use modality of the system. A large screen is helpful in creating forms for the experiments and import or export it from/to excel. The data-logger is equipped with a bar code reader. It is used to gather, update and view all data in real-time, in the labs and outside (greenhouse, field, etc.). A Local Area Network connects the data-loggers and desktop workstations to the system server. The connection is ensured by wireless lan. In this way mobile devices (data-loggers) exchange information with DB server and, by means of a label scanner, allows a direct access to all data previously stored in database. The mobile printer prints bar code labels that uniquely identify the objects (microorganism, chemical, record of the experiment).

The application has a very simple and friendly interface for both versions of the system; each user has a login and a password to access the system, with three different levels: administrator (full access), regular user (most of the menus) and restricted user (excludes access to confidential information). The data base is accessible from desktop and mobile version as a general form, with several fields for each record. LEXMas diagnoses input errors and it explains the support the users by explaining what needs to be fixed.

LEXMaS functionality

The system can be used as a tool for collecting organised data, and also for viewing safety information connected with each object in the category of chemicals and microorganisms.

Chemicals’ data management

All the information on each single chemical can be stored (molecular formula and weight, supplier, quantity in stock, etc.). The system allows identifying the hazardous classes related to the chemical (i.e. irritant, toxic, harmful, etc.). For each single chemical a hyperlink to files with specific procedures for a correct handling and the specific Material Safety and Data Sheet (MSDS) for the chemical or safety data sheet for the microorganism, which provide short instruction to act in emergency cases (fire fighting, accidental release, aid measures, etc.) can be created.
Microorganisms’ data management
For each microorganism the system stores information on identification (genus, species, subspecies, strain, group), isolation (isolation date, name of identifier, substrate of origin, isolation method, site of origin, location, country, GIS coordinates, ownership), activity (type of activity), growth and preservation (culturing method, preservation method, checks of the collection). It can also include the hyperlink to bibliography and safety procedure on how to handle the organisms, in order to reduce the exposure to biological hazard.

Experiments’ data management
The third section of LExMaS allows collection of data related to individual experiments. The system requires the ID (identification) name/number of the experiment and the date in which it starts. A form in which data will be filled in is based on the design of the experiment and it is built according the number and name of dependent and independent variables and the number of sub-levels of each variable. In this way each record can be clearly input and identified.

LExMaS Usage
Implementation of a specific instance of the system includes two steps: data insertion and labels printing. The system can then be updated with modifications of each record, i.e. quantity of chemical in stock and periodical check for microbial collections. Label scanning translates the code in output information that appears on computer desktop or on the data-logger. In this way qualitative and quantitative parameters of each labelled object (chemical, microorganism, sample of an experiment) are available for the user (Fig. 2). After experiment planning and labeling each sample, the user can, with the label scanning, locate the specific record and insert its value in the DB. It is also possible to view the previous values of the samples in the experiment (Fig. 3).

Building a growth curve of a microorganism at different conditions can serve as example. The first step is to set the experiment database, by inputting the identification code (ID number and Experiment starting data), the independent variables (for example three independent variables: temperature, relative humidity and replicates) and the dependent variables (for example two dependent variables: colony size, colony colour).
The second step implies to register the name and the levels of each independent variable (temperature: 25 and 37°C; relative humidity (RH): 50 and 100%; replicates: 5) or dependent variable (colony size: diameter; colony colour: intensity). Obviously, the created data base has been completed after the adding of the data regarding the dependent variable (length obtained after growth or intensity watched). Bar code labels for each growth condition can be printed and fixed on the Petri dishes containing the microorganism. Later on each assessment time the bar code label can be scanned, the system will locate the exact position in the experiment database and the assessment (diameter and colour intensity can be stored). The stored data can be exported in excel to be analyse with any statistic software.

The user can also query the system data base. Queries provide information related to a specific characteristic, for example all the microorganisms in the collection belonging to a certain genus, with a specific activity or isolated in a certain location or year). The LExMaS system implementation in our labs achieved the following feedback from the users: friendly and simple interface, reliable and secured connection to database, good mobility, input errors detection, fast networking, preventing data collisions, valuable queries of data, data import/export (Excel).

**Conclusions**

LExMAss provides long term benefits for lab experiments information management. With the classical approach safety information on a chemical or previous data of an experiment are not easy to find. Even if the data are stored in a computer the process of searching can take time and mistakes are always possible. LExMaS, even working in an area far from the stationary location, guarantees easy data access and reliability. This original application of the bar code
could become the starting point for a continuous improvement of work quality and safety. There are always hazards and risks at work, especially while working with chemicals and microorganisms, so easy access to information is a key point to guarantee safety at work.

The unique identification of each sample in the experiments reduces the risk of mistakes while assessing results. The integration of information and a good data management can be a good formula to improve work quality and safety standards for workers, also including the satisfaction of stakeholders.

Figure 3. Viewing the DB of an experiment related to the microorganism colony growth with two levels of two independent variables (temperature and relative humidity) and two levels of a dependent variable (colony size and colour)

Acknowledgements

We thank Susanna Micheli and Elisa Bozza for technical support. Research was supported by SafeCrop Centre funded by Fondo per la Ricerca, Autonomous Province of Trento.

References

Development of a Real-time PCR method for quantification of *Trichoderma atroviride* 122F in soil and comparison with soil dilution plating and qualitative PCR methods

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**Abstract**: Real-time PCR has been proved to be one of the most powerful techniques for quantification and detection of microorganisms in several areas, i.e. medical diagnosis and GMO detection, due to its high sensitivity, accuracy and specificity. In the present study a method for quantification by real-time PCR is applied for detecting and quantitatively estimating the presence of *Trichoderma* spp. and a biocontrol agent (BCA) *T. atroviride* 122F in soil using specific primers. The method was developed using the criteria of the validation process, i.e. specificity, applicability, sensitivity, dynamic range and accuracy. Its validity in monitoring the BCA presence and quantity after its release in the environment was tested in soil and on plant surface experiments, in microcosms and under field conditions. The method appears to be reliable and specific, but it should be validated and checked with additional experiments using samples from intact soil microcosms and field, where unidentified strains of *Trichoderma* may be present and could interfere with the PCR amplification.

**Key words**: biocontrol agent, diagnosis, risk assessment

**Introduction**

Real-time PCR, currently used to trace and quantify the dispersion of genetically modified organisms (GMOs) in the environment and in the food chain, is becoming an important tool in plant pathology for a rapid detection and identification of pathogens. The sensitivity and the high number of samples that can be processed are only two of the most appreciated features of this technique, considered a useful complement of the traditional ones (Gibson, 2006; Atkins et al., 2003; Schena et al., 2003). The relatively high cost for developing Real-time PCR methods and the need of the DNA extraction had, up to now, limited the application of this technique in soil. Other limitations are the few specific and validated methods for plant pathogen or other microorganisms identification and quantification, in opposite to the large number of validated methods and studies applied to GMO analysis (ENGL, 2005).

In the present study we set up a methodology for the traceability by Real-time PCR of *Trichoderma atroviride* strain 122F, which has a good potential as biocontrol agent of *Armillaria mellea*. The improvement of a Real-time PCR method for specific microorganism traceability and quantification in the environmental conditions where other microorganisms or chemical compounds could interfere with the PCR analysis (Rubio et al., 2005) needs special care to ensure the highest specificity and sensitivity. DNA quantification requires an endogenous reference gene, which must be the calibrator of the genome number of the organism to be quantified. This gene must be accurately chosen. It has to be present in single or known number of low copies in the genome; it should be species-specific, or genus-specific as in our research (*Trichoderma* spp.). The single-copy alpha subunit of heterometric G protein (Tga3) gene (Zeilinger et al., 2005; NCBI Acc. N. AF452097.1) seems to be
common among *Trichoderma* spp., and suitable for balancing and comparing the following strain-specific tool. The strain-specificity was achieved with the finding of several nucleotide mutations in the sequence of the Endochitinase 42 gene (Ech42), on which several primers and probe sets for PCR were constructed and compared with the Tga3 amplification (Mattarucchi et al., 2005; Dodd et al., 2004).

**Material and methods**

**Trichoderma spp. isolates and DNA extraction**

A total of 44 *Trichoderma* spp. isolates were used (Table 1) taking into account the wide genetic diversity spreading in the *Trichoderma* genus. The isolates were cultured on PDA four weeks in the dark at 20°C. DNA was extracted from the mycelium using the DNeasy Plant Mini Kit (Qiagen). DNA quality and quantity was checked on electrophoresis 1% agarose gel in TAE 0.5% solution and SYBR Safe DNA dye. The DNA was stored at -20°C until use.

**Soil Static Microcosm (SSM) and DNA extraction**

Eight polypropylene boxes (Soil Static Microcosms) containing 100 g of sandy soil from S. Michele all’Adige were treated with increasing concentration of *T. atroviride* 122F, ranging from 0-10^6 conidia/g soil, and incubated at room temperature over night (Fig. 1). Soil samples were collected the day after inoculation. Samples of 7.5 g of soil were used for DNA extraction using Power Maxi Soil DNA Isolation Kit (Mo Bio). DNA quality and quantity was checked on electrophoresis 1.5% agarose gel in TAE 0.5% solution and SYBR Safe DNA dye. For the precision test, four soil samples of 200 mg, from each concentration level, were extracted using the Power Mini Soil DNA Isolation Kit (Mo bio) in the same session.

**Qualitative PCR**

The primers sets used in qualitative PCR were 122-G1 and 122-G2 (598 and 471 bp amplicon product size, respectively) developed from RAPD-PCRs (SCAR primers). The mix was composed of the following reagents: Buffer 1× with 2 mM MgCl₂ included, dNTP 0.2 mM, each primer 0.4 µM, 1 U of Taq DNA Polymerase (Eppendorf), 1 µl of the sample, corresponding to 10-100 ng DNA. The 9700 (Applied Biosystem) PCR Thermocycler was used and the amplification cycle consisted of 5 min at 94°C of denaturing followed by 45 cycled of 30 sec at 94°C denaturing, 40 sec at 60°C of annealing and 72°C of extension, with a final extension of 7 min at 72°C. Fifteen µl of amplification product were checked on 1.5% agarose gel in TBE 0.5% with SYBR Gold DNA stain.

**Quantitative Real-time PCR**

Several primer sets and probes were tested in the Real-time PCR, as shown in the Table 2, constructed on the sequences of the Endochitinase42 (Ech42) and of the alpha subunit of heterometric G protein (Tga3) genes, obtained from the *T. atroviride* 122F strain and compared with the sequences presents in NCBI Data Bank with BLAST programme and CLUSTALW (www.ncbi.nih.gov/ and www.ebi.ac.uk/clustalw/). A standard curve was built with 1:3 serial dilutions of *T. atroviride* 122F DNA, at known quantity. The Real-time PCR apparatus used was the PTC-200 Chromo 4 Thermocycler (MJ Research); the analysis of the amplification, standard and melting curves was performed with the Opticon Monitor2 Program (MJ Research).

**SYBR Green I Real-time PCR**

The amplification mix was composed of the following reagents: Buffer AB SYBR Green I (Applied Biosystem) 1× with MgCl₂, dNTPs and Taq included, 0.3 µM each primer and 1 µl of the sample. The PCR conditions were 10 min at 95°C of denaturing- Hot Start followed by
45 cycles of 15 sec at 95°C of denaturing and 1 min at 60°C of annealing/extension, with a final dissociation (melting) from 50 to 90°C of 10 sec each 0.5°C.

Table 1. *Trichoderma* species, collection and country of origin

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection and Isolate code</th>
<th>Origin</th>
<th>Species</th>
<th>Collection and Isolate code</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. agressivum</em></td>
<td>CBS 115901</td>
<td>Israel</td>
<td><em>T. viride</em></td>
<td>CBS 456.96</td>
<td>Netherlands</td>
</tr>
<tr>
<td><em>T. atroviride</em></td>
<td>CBS 101052</td>
<td>Netherlands</td>
<td><em>T. reesei</em></td>
<td>CBS 102271</td>
<td>Brazil</td>
</tr>
<tr>
<td><em>T. breviciputum</em></td>
<td>CBS 112447</td>
<td>Mexico</td>
<td><em>T. reesei</em></td>
<td>CBS 392.92</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. hamatum</em></td>
<td>CBS 101531</td>
<td>Netherlands</td>
<td><em>T. reesei</em></td>
<td>CBS 820.91</td>
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</tr>
<tr>
<td><em>T. harzianum</em></td>
<td>CBS 226.95</td>
<td>UK</td>
<td><em>Trichoderma</em> spp. Safecrop 9F</td>
<td>Italy</td>
<td></td>
</tr>
<tr>
<td><em>T. koningii</em></td>
<td>CBS 141.96</td>
<td>Netherlands</td>
<td><em>Trichoderma</em> spp. Safecrop 70F</td>
<td>Italy</td>
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<td><em>T. longibrachiatum</em></td>
<td>CBS 339.92</td>
<td>India</td>
<td><em>Trichoderma</em> spp. Safecrop 2918</td>
<td>Italy</td>
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<td><em>T. longipile</em></td>
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<td>Canada</td>
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<td><em>T. polysprum</em></td>
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<td><em>T. pubescens</em></td>
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<td><em>T. reesei</em></td>
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<td>Solomon Island</td>
<td><em>Trichoderma</em> spp. SafeCrop Cl-3B</td>
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<td>France</td>
<td><em>Trichoderma</em> spp. SafeCrop Cl-5C</td>
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<td><em>Trichoderma</em> spp. SafeCrop Cl-6C</td>
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</tr>
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<td><em>T. atroviride</em></td>
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<td>Israel</td>
<td><em>T. atroviride</em></td>
<td>ATCC74058</td>
<td>Unknown</td>
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<tr>
<td><em>T. atroviride</em></td>
<td>CBS 142.95</td>
<td>Slovenia</td>
<td><em>T. harzianum</em></td>
<td>Volcani Center T39</td>
<td>Israel</td>
</tr>
<tr>
<td><em>T. atroviride</em></td>
<td>CBS 347.96</td>
<td>Papua New Guinea</td>
<td><em>T. atroviride</em></td>
<td>Safecrop 122F</td>
<td>Italy</td>
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<td><em>T. viride</em></td>
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<td>Netherlands</td>
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<tr>
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<td>UK</td>
<td><em>T. viride</em></td>
<td>CBS 456.96</td>
<td>Netherlands</td>
</tr>
</tbody>
</table>

Table 2. Primers, probe Sets and amplified genes in the Qualitative and Real-time PCR exps

<table>
<thead>
<tr>
<th>Primers/Probes</th>
<th>PCR</th>
<th>Gene amplified</th>
<th>Processed samples</th>
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<tr>
<td>G1 and G2</td>
<td>Qualitative</td>
<td>Unknown</td>
<td>SSM*</td>
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<tr>
<td>Primers Set 12</td>
<td>Real-time with SYBR Green I</td>
<td>Ech42</td>
<td>SSM</td>
</tr>
<tr>
<td>Primers Set 21</td>
<td>Real-time with SYBR Green I</td>
<td>Tga3</td>
<td>SSM</td>
</tr>
<tr>
<td>Primers and Probe</td>
<td>Real-time with SYBR Green I</td>
<td>Ech42</td>
<td>SSM and <em>Trichoderma</em> spp. isolates</td>
</tr>
<tr>
<td>Set SNP2-3</td>
<td>and TaqMan MGB probe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer and Probe</td>
<td>Real-time with SYBR Green I</td>
<td>Ech42</td>
<td>SSM and <em>Trichoderma</em> spp. isolates</td>
</tr>
<tr>
<td>Set SNP9-10-11</td>
<td>and TaqMan MGB probe</td>
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<td></td>
</tr>
<tr>
<td>Primer and Probe</td>
<td>Real-time with SYBR Green I</td>
<td>Tga3</td>
<td>SSM and <em>Trichoderma</em> spp. isolates</td>
</tr>
<tr>
<td>Set L</td>
<td>and TaqMan MGB probe</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Static soil microcosm*
**TaqMan-MGB (Minor Groove Binder) Probe Real-time PCR**
The amplification mix was composed of the following reagents: Buffer IQ Multiplex PowerMix (Bio-rad) 1× with MgCl₂, dNTPs and Taq included, 0.3 µM each primer, 0.2 µM each probe and 1 µl of the sample. PCR conditions were 2 min at 95°C of denaturing followed by 45 cycles of 15 sec at 95°C of denaturing and 1 min at 60°C of annealing/extension.

**Results and discussion**

**Qualitative and Real-time PCR sensitivity comparison**
The sensitivity of both the qualitative and Real-time PCR was evaluated amplifying the same quantity of DNA extracted from the static microcosm experiment. The Agarose Electrophoresis gel of the qualitative PCR amplification and the Real-time PCR quantification (performed using SYBR Green I as fluorescent dye) are presented in the Fig. 1. The qualitative PCR could reveal only the highest concentrations of BCA (10⁵ and 10⁶ conidia/g), while the Real-time PCR detected and quantified up to the DNA concentration level 4 corresponding to 10² conidia/g. The Melting Curve, showed a unique single peak at 80°C, revealing the lack of a primer-dimerization and the unique amplification event with both the primer sets used in this experiment (set 12 and 21).

**Specificity for the T. atroviride 122F strain**
Several primers and probe sets were constructed on the nucleotide mutations found in the Endochitinase42 gene sequence (Ech42) of the 122F strain. A second group of primers-probe sets were built on the Tga3 gene sequence, a single copy gene, within a common sequence among several isolates of *Trichoderma*. The Primer sets were previously checked in Data Bank for their specificity to 122F strain (Ech42) and for *Trichoderma* spp. specificity (Tga3). Several Real-time PCR amplifications with the different Primer-Probe Sets were carried out to find the best specificity for the strain 122F and the better fluorescence results: the Ech42 Sets constructed on the nucleotide mutations were tested on the available *Trichoderma* spp. (see Table 1), in comparison with the Set on Tga3 gene, which should amplify all the strains, thus representing a positive control amplification. The results are very satisfying: one Primer-probe Set (SNP9-10-11) amplifies only two strains from CBS collection (*T. reesei* and *T. longipile*) on 44 total strains tested. Moreover, both the amplifications have the same Ct (Threshold Cycle) in the 122F strain, meaning that the Ech42 amplicon is in single copy in the *Trichoderma* genome, as the Tga3 amplicon (Data not shown).

**Precision test of the Real-time Quantification**
Four DNA extractions of 200 mg of soil with five conidia concentrations (0, 10¹, 10³, 10⁵ and 10⁶) were quantified by Real-time PCR, using both SYBR Green I and TaqMan MGB Probe as fluorescent dye. The results (Fig. 4 and Table 3) show, as expected, that the Real-time PCR gave a better accuracy as the comparison between the expected and observed results, in levels 10³-10⁵, particularly with the Ech 42 primer set and the TaqMan probe dye. Moreover the precision, as the repeatability of the relative standard deviation is higher in the middle levels of concentrations, with 20-40% RSDr, and lower for the most concentrated DNA samples (10⁶) (Data not shown). These results revealed that the method of DNA extraction and the Real-time PCR gave a satisfactory precision and accuracy, even if some modifications in the primer set and probes should be necessary.
Figure 1. A: Agarose gel electrophoresis of the G1 Primer Set amplification (598 bp amplified band); M: 1Kb DNA Marker (Promega), 1= negative control (water), 2-8 lanes: soil samples (conc. 0-10^6 conidia/g). B: Amplification curves of samples 1-8 for primer set 12 (Ech42).

Table 3. Quantification of the Trichoderma atroviride F122 haploid genomes in five soils microcosms amended by various quantities of conidia

<table>
<thead>
<tr>
<th>Level</th>
<th>N° released conidia/g soil</th>
<th>10^3 copy number of haploid genome / g soil</th>
<th>and threshold cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sybr Green I Set 12 (ech42)</td>
<td>Sybr Green I Set 21 (tga3)</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>10^1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>10^3</td>
<td>0.24 (0.57)</td>
<td>1.5 (1.1)</td>
</tr>
<tr>
<td>7</td>
<td>10^5</td>
<td>90 (0.22)</td>
<td>239 (0.3)</td>
</tr>
<tr>
<td>8</td>
<td>10^6</td>
<td>75 (0.51)</td>
<td>292 (1.39)</td>
</tr>
</tbody>
</table>

Results are the average of four DNA extractions, with the exception of the samples *, where the data is the average of only one sample amplified. The results are shown as copy number of haploid genome of Trichoderma spp./g soil. Values in () are the standard deviation.

In this study we proposed two isolate-specific Real-time PCR quantification methods for Trichoderma spp. and for the T. atroviride 122F. Both provided good features of reliability for genus and strain specificity, and they can detect and quantify the fungus also in difficult matrices such as soil samples. The method appears to be reliable and specific, but it should be validated and checked with additional experiments using samples from intact soil microcosms and field, where unidentified strains of Trichoderma may be present and could interfere with the PCR amplification.
Real-time PCR equipment is expensive at the moment, the methodology improvement for a specific case is time consuming, highly skilled personnel is required, and the cost for a single comparable analysis is higher compared to classical methods. Time needed for a single analysis (DNA extraction, amplification, interpretation of the results) is comparable to the classic method (serial dilution of soil sample, plating on medium, colony counting). Therefore Real-time PCR can be competitive with classical methods only with the automation of the procedure and with the analysis of a large number of samples. The accuracy of the Real Time PCR can be considered higher since the human errors are reduced (the part of counting colonies is avoided). The small dimension of soil samples can pose problems in selecting representative samples, but can be overcome enlarging the number of replicates in high variable soils. In spite the higher equipment and development costs Real-time PCR based methods can be therefore more suitable when it is necessary to run routine assessments to identify a precise strain. With classical methods, if a “strain selective medium” is not available, a qualitative PCR of each single colony is needed. The present Real-time PCR based methods can be preferably used, for example, to check if the level of the organism is increasing or decreasing in natural conditions. Moreover the method cannot distinguish alive and dead conidia, but it is measuring non-degraded microorganism’s DNA.

Acknowledgment

This research was supported by SafeCrop Centre, funded by Fondo per la Ricerca, Autonomus Province of Trento.

References


Production and formulation to improve activity
Improvement of biocontrol of *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium* spp. by formulation of *Penicillium oxalicum*

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Abstract: Sugars, polyalcohols, inorganic salts, and detergents were added to conidia of *Penicillium oxalicum* at three different points of the production-formulation process to improve water dispersal. Effects also were tested on conidial germination and production. Conidial dispersal in water improved when 1.5% sodium alginate was added to the substrate in bags before production, and when 1.5% sodium alginate, 60% sucrose, 60% D-sorbitol, 60% fructose, 5 to 20% PEG 8000, or 20% glycerol were added to conidia before drying. Several *P. oxalicum* formulations significantly reduced tomato wilt caused by *Fusarium* spp. under greenhouse conditions and by *Verticillium* spp. in a field assay. These formulations were then evaluated against tomato wilt in three glasshouse (G1 to G3) and two field (F1 and F2) experiments. A range of 22–64% of disease reduction was observed with all formulations. Our results provide an effective method to add additives to the conidial production process or during formulation as a dry product to improve *P. oxalicum* dispersal, increasing disease reduction from wilt of tomato caused by *Fusarium* and *Verticillium* spp.

Key words: biofungicide, biological agent

Introduction

*Penicillium oxalicum* Currie & Thom is a promising fungal agent for biological control of soilborne diseases of tomato (De Cal & Melgarejo, 2001; De Cal et al., 1995). Application of a conidial suspension of *P. oxalicum* by watering the tomato seedlings in seedbeds 7 days before transplanting usually results in a significant reduction of *Fusarium* and *Verticillium* wilts of tomato plants (De Cal et al., 1999). Induction of resistance in tomato plants was demonstrated as the main mode of action of *P. oxalicum* against *Fusarium oxysporum* f. sp. *lycopersici* (De Cal et al., 1997). Conidia of *P. oxalicum* must contact the tomato roots (De Cal et al., 2000, 1997) and a range of $10^6$ to $10^7$ conidia g$^{-1}$ in seedbed substrate and rhizosphere before transplanting is required for effective control of wilts of tomato (Larena et al., 2003a).

*P. oxalicum* conidia prepared by fluid bed-drying reduced the incidence of tomato wilt under glasshouse and field conditions (Larena et al., 2003b). However, an important problem associated with applications of *P. oxalicum* is the hydrophobic nature of conidia, especially as dried conidia. The objective of the present study was to evaluate the effect of different additives for dispersal of dried *P. oxalicum* conidia in water, in order to develop new biological formulations against tomato wilt.
Material and methods

Cultures
Conidia of *P. oxalicum* (PO) (ATCC No 201888) were produced in a solid-state fermentation system and dried in a fluid bed dryer (Larena et al., 2002; Larena et al., 2003b). Chlamydospores of *F. oxysporum* f. sp. *lycopersici* (FOL) (ATCC No 201829) were produced in bags (80×40×20 cm³) containing sterile peat inoculated with a microconidia (10⁵ microconidia g⁻¹) formulation of peat, and left in the glasshouse for 30 days at 20 to 30ºC.

Plant material
Tomato cv. San Pedro, Muchamiel and Valencia1 susceptible to races 1 and 2 of FOL and to *Verticillium dahliae* (VD), were used in all experiments.

Additives
Several compounds such as sugars, polyalcohols, inorganic salts and detergents were used due to their features as dispersants, binders, humectants, emulsifiers, and wetters. Additives were dissolved in distilled water wt:vol (solids) or vol:vol (liquids) and then autoclaved at 1.0 kg cm⁻² at 120ºC for 20 min. Additives were tested for toxicity (effect on conidial germination and production) against PO at several doses. Formulations of PO conidia were obtained after addition of one or various additives in two separate steps of the production and drying of PO conidia: (i) to substrate in bags before production, and (ii) to conidial paste obtained after production but before drying.

Dispersal assays
Dispersal of conidial suspensions was estimated by measuring absorbance at 420 nm in a spectrophotometer. In each experiment, the percentage of increase of conidial dispersal was calculated by the formula ([(dispersal of conidia with additive – dispersal of conidia without additive]/[dispersal of conidia without additive])×100.

Glasshouse and field experiments
Three glasshouse experiments were carried out to study the biocontrol efficacy of eight different formulations of conidia of PO against tomato wilt. All treatments were made to give 6×10⁶ conidia g⁻¹ of seedbed substrate. The experiment was established in a randomized complete block design with four replications. Each treatment had a plot in each replicate block. The association of FOL or VD with disease symptoms in all plants was examined. Experiment G1 and G2 were carried out in 2003 and 2004 in commercial plastic glasshouses located in eastern Spain. Tomato seedlings were transplanted in plots previously treated with PO formulations. The control treatments consisted of: (i) plants treated with the reference fungicide LAICON-L (0.04 mg ml⁻¹ Polioxine-B p/v SL, Laicon, Barcelona, Spain) (FU), and (ii) non-treated plants (NT). Disease incidence was recorded at the end of the experiment based on the plants with tomato wilt symptoms. Experiment G3 was similarly performed in an experimental plastic glasshouse with artificial soil and temperature control located in Madrid (Spain) in 2004. Seedlings were transplanted into sterile peat bags, which were previously inoculated with FOL as previously described.

Two field (F1 and F2) experiments were carried out during 2003 and 2004, respectively, in two fields located in Aranjuez (Madrid, Spain), where VD wilt disease had been prevalent in previous years. Both experiments were established in a design with five or four replications (plots) per treatment in 2003 and 2004, respectively. Ten tomato seedlings (cv. San Pedro) per block and treatment were transplanted in 12 m² plots. Tomato plants were treated in seedbeds with the formulations of PO. The control treatment consisted of plants without PO (NT). Disease incidence was recorded at the end of the experiments (130 days after transplanting) based on wilt symptoms.
Results and discussion

The best results on dispersal of conidia were obtained when additives (glycerol, PEG 8000, sodium alginate, and sugars) were added to conidia before drying (Fig. 1). These compounds form hydrogen bonds with functional groups of protein molecules on dry conidia surfaces (Pascual et al., 2000) and allow rehydration and improve dispersal by reducing the hydrophobicity of conidial surfaces of PO. Conidial dispersal also was enhanced when sodium alginate was added to the substrate (Fig. 1). These studies demonstrate that adding additives to the growth substrate significantly improved the ability of conidia of PO to disperse in water.

![Figure 1. Effect of different additives added before production (A), before drying process (B), and to dry conidia, on conidial dispersal of Penicillium oxalicum](image)

But not all PO formulations showed the same percentage of disease control against similar tomato wilt incidence (Fig. 2). Discrepancies in the efficacy of biological treatment between controlled conditions and commercial situations have been attributed to uncontrolled environmental conditions and their effects on the biocontrol agent. But similar discrepancies in disease control have been observed in tomato plants treated with LAICON-L fungicide: it reduced tomato wilt only in a one of the glasshouse experiment. Basic environmental conditions and soil physical and chemical characteristics can greatly affect the physiology of the host plant and subsequent disease development, as well as alter the interactions between plant and pathogen, and the relation between establishment, survival, and activity of the biocontrol agents.

Formulations were also efficient independently of tomato cultivars (without inherent genetic resistance). It was also demonstrated that different tomato cultivars with different inherent genetic resistance to fusarium wilt showed a reduction in fusarium wilt when they were induced by PO in growth-chamber assays (De Cal et al., 1999b). PO-mediated reactions observed in infected tomato plants reflect a generalized resistance response rather than a specific induction of resistance against a given pathogen (Larena et al., 2001, 2003a), on different cultivars of tomato (De Cal et al., 1999b).
Figure 2. Percentage of disease incidence caused by FOL or *Verticillium dahliae* on tomato plants at the end of glasshouse (A) and field experiments (B) after application of different PO formulations. Treatments were: FOR1, addition of 60% sucrose before drying; FOR2, addition of 1.5% sodium alginate before drying; FOR3, conidial produced with 1% Tween 80 + 0.1% skim milk; FOR4, conidia produced with 10% NaCl; FOR5, conidia produced with 1.5% sodium alginate and addition of 60% sucrose before drying; FOR6, addition of 20% PEG8000 before drying; FOR7, addition of 10% calcium chloride before drying; and FOR8, conidia produced with 1.5% sodium alginate and addition of 1.5% sodium alginate before drying. FU = LAICON-L (0.04 mg ml⁻¹ Polioxine-B p/v SL). NT, non-treated plants.

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References


Optimisation of the freeze-drying process of
Pseudomonas fluorescens strains Pf 153 and CHA0

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Abstract: Within the development of a freeze-drying protocol the freezing process and the drying temperature were optimised for the Pseudomonas fluorescens strains Pf 153 and CHA0. When 21 different protectants were compared, especially sugars protected the vegetative cells during the freeze-drying process. After the optimisation of the process the viability before and after freeze-drying of Pf 153 was not significantly different. Therefore, these results demonstrate that the desiccation sensitive P. fluorescens can survive a drying process without loss of viability. Additional storability tests were carried out with the best protectants: skimmed milk, sucrose, glucose, lactose and lignosulfonic acid. The highest viability after storage was obtained when Pf 153 and CHA0 were formulated in lactose. When the efficacy of freshly produced and freeze-dried pseudomonads were compared ad planta, in different plant-pathogen systems, no significant differences were obtained. But, depending on the pathogen, the protectants were influencing the efficacy of the pseudomonads.

Key words: formulation, lyophilisation, storability

Introduction

The formulation and application of micro-organisms are important steps within the development of biocontrol agents. Although it has been demonstrated that Pseudomonas fluorescens is an effective antagonist for control of several plant diseases, limited appropriate formulations are available. The most critical point in the formulation of pseudomonads is the limited viability of formulated cells and their storability. One common method to conserve micro-organisms is freeze-drying. But up to now no freeze-drying protocols are available for pseudomonads. Therefore, research was carried out on the development of freeze-drying protocols for Pseudomonas fluorescens strain Pf 153 and CHA0.

Material and methods

Both P. fluorescens strains were obtained from the ETH, Zürich, CH. For the pre-culture an aliquot of 25 ml of nutrient broth was inoculated with a slope of culture grown on TSA and was incubated for 72 hours on a horizontal shaker (Novotron, Infors, Switzerland) at 28°C and 150 rpm. For the main culture 250 ml Erlenmeyer flasks with 100 ml of nutrient broth were inoculated with 1 ml of the pre-culture and were incubated at 28°C and 150 rpm.

The micro-organisms were harvested after 24 hours cultivation when they reached the stationary growth phase. The liquid culture was centrifuged for 5 min at 8250 g (Biofuge, Heraeus, Germany) and the pellet was re-suspended with phosphate buffer (pH 7.0). Each sample was centrifuged three times. The bacterial suspensions were adjusted to an optical
density of 0.95 (±0.05) using a Kontron UVIKON 922 spectrophotometer. The viability of the micro-organisms was determined by the most probable numbers (MPN) method calculated with the Most Probable Number Calculator (Version 4.04© 1996, United States Environmental Protection Agency, Cincinnati, USA).

**Freeze-drying process**

1.5 ml samples were mixed with 1.5 ml of an autoclaved or boiled solution of the protectant and were transferred in 10 ml vials. Afterwards, the suspension was freeze-dried in an Advantage EL (VirTis, Gardner, USA) freeze-drier. During the freeze-drying process the temperature of the product was measured. For the storability experiments the vials were closed under vacuum in the freeze-dryer.

**Comparison of different protectants**

In total 21 different protectants were compared. For this a solution/suspension of the protectants was autoclaved for 10 min at 115°C. Albumin, egg yolk, lecithin, soluble starch and skimmed milk were boiled twice in a microwave at 600 W. After cooling down to room temperature the suspension of Pf 153 was mixed 1:1 with the protectant and was freeze-dried (1.3 to 1.9°C/min freezing rate, 5°C freeze-drying temperature at 0.2 mbar vacuum for 18h). After freeze-drying sterile and deionised water was added to the dried material until the weight before drying was reached.

**Bioassays**

For soil borne diseases, the cells of Pf 153 were tested against *Pythium ultimum* on cucumber seeds. The experimental design was similar to the description of Koch et al. (1998). For control of leaf diseases, experiments were conducted with Pf 153 against *Phytophthora infestans* on detached potato leaves and against *Botrytis cinerea* on detached *Vicia faba* leaves. The experiments on detached potato leaves of the *P. infestans* susceptible variety Leyla were conducted as described by Stephan et al. (2005). The area under disease progress curve (AUDPC) was determined by measuring the infected leaf area from the fifth to the ninth day. The experimental design of the experiments against *B. cinerea* on detached *Vicia faba* leaves of the variety “Con Amore” was similar to the potato experiment. The boxes were incubated at 20°C and the affected leaf area was measured daily over a period of five days. The AUDPC was statistically analysed. In all bioassays three time independent experiments were conducted.

**Results and discussion**

When PF 153 and CHA0 were freeze-dried under not optimised conditions the survival rate was 4.0 and 15.0%, respectively. For the optimisation of the freeze-drying process different freezing rates and drying temperatures were compared. For Pf 153 the best freezing rate was 0.04-0.12°C/min, whereas for CHA0 1.3-1.9°C/min. The viability was lower when the strains were frozen in liquid nitrogen or with a freezing rate of 0.6-0.8°C/min. Additionally, different drying temperatures were compared for the strain Pf 153. The best results were obtained by drying at 20°C, compared to 5 and 30°C. The results indicate that within the process optimisation possibly for each strain specific freeze-drying parameters have to be identified. After optimisation of the process for Pf 153 no significant differences in the viability before and after freeze-drying were obtained.

When the influence of different protectants on the viability of the strain Pf 153 after freeze-drying were compared, the highest viability was achieved by suspending Pf 153 in skimmed milk, glucose, lignosulfonic acid, lactose or saccharose (Fig. 1). These protectants were chosen for storability tests. For both strains the best storability was obtained by using
lactose as protectant (Fig. 2). The storability was lower, when both strains were freeze-dried with lignosulfonic acid as protectant. Therefore, especially for the two tested strains sugars are interesting protectants for freeze-drying with good storability characteristics.

![Figure 1. Influence of the protectant on the viability of Pf 153 after freeze-drying](image1)

![Figure 2. Comparison of the storability of Pf 153 (left) and CHA0 (right) at 40°C](image2)

The efficacy of freshly produced and freeze-dried pseudomonades was tested in different test systems. In all test systems the efficacy of freeze-dried cells was comparable to the efficacy of freshly produced cells (Fig. 3 and 5). When Pf 153 was formulated with various protectants and tested against *P. infestans* the different sugars did not influence the efficacy. But lignosulfonic acid showed a clear effect on *P. infestans* (Fig. 4). Therefore, lignosulfonic acid can be an interesting additive, especially because it also can be used as UV protectant. When Pf 153 was tested against *B. cinerea* the efficacy was influenced by the protectants (Fig. 5). Although the viability before and after freeze-drying was not significant different, saccharose influenced the efficacy negatively. The results indicate that the selection of the right protectant is also influenced by the pathogen.
The results on the formulation of pseudomonads demonstrate that freeze-drying is an interesting technique for the conservation of *P. fluorescens*. The results also indicate that the selection of the right protectant influences the viability, storability and efficacy. Based on freeze-dried pseudomonads further investigations have to be carried out to develop practicable formulations (e.g. granules or wettable powders).

**Figure 3.** Efficacy of not freeze-dried and freeze-dried Pf 153 cells against *P. ultimum* on cucumber

**Figure 4.** Efficacy of Pf 153 formulated with different protectants against *P. infestans* on detached potato leaves

**Figure 5.** Efficacy of Pf 153 before and after freeze-drying against *B. cinerea* on detached *Vicia faba* leaves

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References


Biological control of snow mould in cereals by a dry formulated pseudomonad

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Abstract: Snow mould (Microdochium nivale) causes considerable losses in rye and winter wheat under Scandinavian climate conditions and heavy chemical treatment is often used to control the disease. Concern for human health and the environment, combined with restrictions on the use of chemical control agents have increased interest in the development of biological control as an alternative to fungicidal use. Healthy seed maintenance is a core problem in organic farming. Biological control products could also offer a good solution to secure healthy seed supply for organic farmers. There are several reports on disease control effects shown by Gram-negative Pseudomonads, however, there are obvious obstacles to develop and process this group of bacteria into a product, which meet the requirements of being user-friendly, having low production costs and being storable with retained and consistent biocontrol effect. These issues are emphasized within the Swedish research program ‘Domestication of Microorganisms for non-Conventional Applications’ (DOM), where domestication tools for biological disease control products is one main study area.

An isolate of Pseudomonas brassicacearum, showing significant effects against snow mould in winter cereal field experiments, was selected as a gram-negative model organism. Basic protocols for fermentation and preparation of a dry formulation of the bacterium were established and evaluated in initial studies. Different dry-formulation protocols based on trehalose were developed and tested for survival rate of the bacterial cells after drying when compared to cell density before drying. The strain was fermented, vacuum-dried and subjected for bioassays in greenhouse experiments. Additionally, the dry formulated product was tested for viability and biocontrol effect after storage. Adding certain additives to the trehalose-based dry protectant gave a dramatic positive response on the survival rate of the dried bacteria. The biocontrol efficacy was retained after re-hydration of dried cells. The dried cells proved to be storable for several months with no reduction of survival rate.
Improving desiccation response and heat shock tolerance of the biocontrol agent *Pantoea agglomerans* CPA-2 by osmotic treatments

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Abstract: Improvement in osmotic and thermal tolerance of *P. agglomerans* cells by modifying growth media with the ionic solute NaCl was achieved. Improved cells exhibited also better survival rates than control cells during spray-drying and fluidized bed-drying processes, and maintained their biocontrol efficacy against postharvest fungal pathogens in apples and oranges. The compatible solutes glycine-betaine and ectoine play a critical role in environmental stress tolerance improvement. This research suggests that it is possible to improve the stress tolerance of the microorganism and thus its behaviour under non-controlled environmental conditions and/or during its formulation process without affecting its biocontrol potential.

Key words: biological control, compatible solutes, cross-protection, ectoine, environmental stress, fluidized bed-drying, glycine-betaine, spray-drying

Introduction

A major hurdle in the exploitation of biocontrol agents is the limited tolerance to fluctuating environmental conditions and the difficulties in developing a shelf-stable formulated product as effective as fresh cells. Most microorganisms are very sensitive to drying processes involved in formulation. Microorganisms in general are able to survive environmental stress conditions by induction of specific or general protection systems, such as cytoplasmatic accumulation of endogenous reserves (compatible solutes). Subjection to a mild stress makes cells resistant to a lethal challenge with the same stress condition and/or can also render cells resistant to other stress conditions (cross protection). We proposed to take advantage of this capacity to improve the behaviour during formulation and practical application of *P. agglomerans* (CPA-2), which is effective to control the main postharvest diseases of pome fruits and citrus. The main aims of this research were to determine the effect of modifying the water activity ($a_w$) of *P. agglomerans* growth medium on (1) water stress tolerance, (2) heat shock survival, (3) desiccation tolerance during spray-drying and fluidized-bed drying processes, and (4) intracellular accumulation of compatible solutes in cells.

Material and methods

**Microorganism and basal medium**

*P. agglomerans* (CPA-2) was obtained from UdL-IRTA Centre, Catalonia, Spain, and had been isolated from apple surface. The basic medium used in this research consisted of sucrose (10 g l$^{-1}$) plus yeast extract (5 g l$^{-1}$) with a pH of 6.7 and a water activity ($a_w$) of 0.995.
Evaluation of viability of *P. agglomerans* in unstressed and water-stressed liquid media

The microorganism was cultured in unmodified liquid (control) or in \( a_w \)-modified (with different solutes) media, and viability of these cells was evaluated on unstressed (0.995) and 0.96 \( a_w \)-stressed solid media, in order to check total viability and \( a_w \)-stress tolerance, respectively. The modified media were prepared at 0.98, 0.97 and 0.96 \( a_w \) by the addition of NaCl (Na 0.98, Na 0.97, Na 0.96), glycerol (Gly 0.98, Gly 0.97, Gly 0.96) and glucose (Glu 0.98, Glu 0.97, Glu 0.96).

Survival of NaCl \( a_w \)-adapted cells to heat shock

The bacterial cells from the unmodified or NaCl osmotically modified treatments (0.98, 0.97 \( a_w \)) were grown 24 h, cells were harvested by centrifugation and resuspended in 2 ml phosphate buffer. Heat treatments were carried out in a water bath at 45ºC. A 5 ml tube of phosphate buffer of each replicate and treatment was immersed in the water bath and once temperature stability had been achieved, inoculated with the treatment bacterial suspension in order to achieve a concentration of \( 1 \times 10^8 \) cfu ml\(^{-1} \). Cells were homogenised, and immediately (0 h) and 30 minutes after inoculation respectively, aliquots of 0.05 ml were taken in order to determine cell viability.

Effect of NaCl-induced resistance on cell survival after spray-drying

Cells grown in NaCl modified media which showed the best low \( a_w \) adaptation were tested in spray drying trials following the method optimized by Costa et al. (2002).

Effect of NaCl-induced resistance on cell survival after fluidized bed drying

*P. agglomerans* was grown on unmodified and NaCl-modified media and centrifuged. Cell paste was mixed with potato starch (carrier) in order to get dough suitable to be manipulated. The dough was mixed thoroughly and extruded through steel nozzles in a length of 0.3-0.5 cm and then dried in a fluidized bed-dryer at 40ºC during 20 min. Bacterial survival (cfu g (dry weight\(^{-1} \)) and final moisture content were determined before and after the drying process.

Determination of osmo-protectants

Suspensions of *P. agglomerans* cells grown in each treatment were centrifuged. The bacterial pellets were resuspended in HPLC grade water and centrifuged again to remove any residual liquid medium. Cells were harvested and freeze-dried. Subsequently, 25 mg cell material was extracted using the method described by Kunte et al. (1993) for quantitative analysis.

Glycine-betaine and ectoine were analysed and quantified by HPLC using a RP8-NH\(_2\) column (125×4 mm filled with 3 \( \mu m \) GROMSIL AMINO 100 RP-8) and a RI Detector. To determine intracellular accumulation of L-glutamine, L-aspartic acid, L-glutamic acid, L-glycine, L-serine, L-alanine, L-proline, L-lysine and L-cadaverine, samples and standards were reacted with 9-fluorenyl-methylchloroformate (FMOC) according to the Einarsson (1985) method with modifications. Amino acids were monitored using a fluorescence detector and a 150×3.9 mm 4\( \mu m \) NovaPack C\(_{18}\) 60 Å column.

Biocontrol efficacy trials

Finally, efficacy of low-\( a_w \) adapted cells was tested on artificially wounded apples and oranges against *Penicillium expansum* and *P. digitatum*, respectively.

Results and discussion

Evaluation of viability of *P. agglomerans* in unstressed and water-stressed liquid media

Cells grown for 24 h in NaCl 0.98 \( a_w \) or for 48 h (Fig. 1) in NaCl 0.98 \( a_w \), 0.97 \( a_w \) and 0.96 \( a_w \), glucose 0.97 \( a_w \) and glycerol 0.97 \( a_w \) showed improved \( a_w \)-stress tolerance in comparison with control cells. The best results were obtained with NaCl treatments (0.98 \( a_w \) and 0.97 \( a_w \)).
Survival of NaCl 

Cells grown in ionically-amended media not only had better tolerance of low $a_w$ but also showed better survival under heat stress. Bacterial cells grown in NaCl 0.98 and NaCl 0.97 media survived 3-log and 1.5-log respectively more than control cells when they are subjected to 45°C for 30 minutes.

It is well documented that transient conditioning to a sub-lethal stress (habituation) can induce tolerance to a more extreme stress and that habituation to one type of stress may cross-protect against others. Generally, during field exposure and/or drying processes used to obtain commercial formulations, biocontrol agents are exposed and suffer a combination of osmotic and thermal stress. Our research has demonstrated that *P. agglomerans* cells grown in characterised ionic-based media can provide a method for better survival under heat challenge than freshly prepared control cells.

**Figure 1.** Viability of *P. agglomerans* cells grown in different liquid media on unstressed (0.995 $a_w$) and stressed (0.96 $a_w$ NaCl) agar-based media after incubation at 30°C for 48h. Statistical analysis was performed within stressed or unstressed solid medium and bars labelled with the same letter are not significantly different according to LSD Test ($P<0.05$).

**Effect of NaCl-induced resistance on cell survival after spray-drying**

Significant differences between growth treatments were found with respect to the survival of spray-dried cells (Fig. 2). The best survival was achieved with cells grown for 48 h in NaCl 0.97 medium (29%), followed by cells grown for 48 h in NaCl 0.98 (23%). The survival rate of control cells was always less than 7%. This represents an improvement, however, it is not sufficiently good in practical terms to consider spray-drying as an appropriate strategy for dehydrating this biocontrol agent.

**Effect of NaCl-induced resistance on cell survival after fluidized bed drying**

Improved cells also exhibited better survival rates than control cells during fluidized-bed drying process with more than 1-log improved for all NaCl treatments. In this case final
formulated products showed viabilities around $1 \times 10^{11}$ cfu g$^{-1}$ which is a more reasonable concentration for a commercial product.

**Determination of osmo-protectants**

HPLC analysis showed that NaCl modified cells accumulated substantial amounts of the compatible solutes glycine-betaine and ectoine in contrast to the control cells, which contained little or none of these two compounds (Fig. 3). Both compatible solutes play a critical role in environmental stress tolerance improvement of *P. agglomerans*. A direct relation between FMOC amino acids accumulated and improved stress tolerance was not observed.

![Figure 2](image.png)

**Figure 2.** Effect of growth media and incubation time (24 h ■ and 48 h □) on the survival of *P. agglomerans* cells after spray-drying. Columns with the same letter are not significantly different ($P < 0.05$). Vertical bars indicate standard deviations.

![Figure 3](image.png)

**Figure 3.** Accumulation of intracellular glycine-betaine ( ■) and ectoine (✎) in *P. agglomerans* grown for 24 h at 30°C in unmodified medium (control) or medium modified with NaCl to 0.98 and 0.97 $a_w$. 
**Biocontrol efficacy trials**

The efficacy of unmodified and the two sodium chloride modified (0.98 and 0.97 $a_w$) treatments was not statistically different with reductions of rot incidence higher than 77% and 73% observed on artificially wounded and inoculated apples and oranges, respectively.

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**References**


Control of post-harvest diseases on citrus using additives to improve biocontrol activity of *Pantoea agglomerans* CPA-2 in pre-harvest applications

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**Abstract:** To increase the adherence and persistence of the biocontrol agent *Pantoea agglomerans* CPA-2 on oranges surface, different additives were added to this antagonist and sprayed on detached oranges. The population dynamics study showed that one of the additives called Food Coat (FC) improved both viability and persistence of the strain CPA-2 in laboratory trials and in pre-harvest applications. Moreover, the pre-harvest application of CPA-2 with FC showed similar efficacy controlling post-harvest pathogens than CPA-2 post-harvest treatment. FC improves adherence, persistence and homogeneity of CPA-2 pre-harvest treatment to protect citrus against post-harvest pathogens.

**Key words:** adherence, biological control, green mold, *Penicillium digitatum*, persistence

**Introduction**

*Penicillium digitatum* is primarily a wound pathogen that can infect fruit in the field, the packinghouse and during distribution and marketing; therefore, it would be advantageous to apply biocontrol agents before harvest, which would reduce initial infection and then remain active and control pathogens in storage and/or commercial conditions. However, biological control at field conditions is usually limited by fluctuating environmental conditions and by the narrow range of environmental conditions in which biocontrol agents are able to survive, establish and effectively control pests and diseases. Some additives could be added to the antagonist in order to improve adherence, persistence and treatment homogeneity.

*Pantoea agglomerans* strain CPA-2 has demonstrated to be effective to control the main post-harvest diseases of pome fruits (Nunes et al., 2002) and citrus (Teixidó et al., 2001). In 2004-05 season, the mentioned bacterium was applied at pre-harvest on orange trees and it was observed that it had survival problems on the orange surface at field conditions and consequently the biological treatment at pre-harvest was not effective to control post-harvest rots (unpublished data). The main goal of this research was to find an additive for improving both viability and persistence of this biocontrol agent at field conditions in order to enhance its efficacy in biocontrol pre-harvest treatments against post-harvest pathogens on citrus.

**Material and methods**

*Microorganism and growth medium*

*P. agglomerans* (CPA-2) was obtained from UdL-IRTA Centre, Catalonia, Spain, and had been isolated from apple surface. Growth medium consisted of sucrose (10 g l⁻¹) plus yeast extract (5 g l⁻¹) with a pH of 6.7 and a water activity (a_w) of 0.995. The biocontrol agent (BCA)
was freeze-dried and formulated following the method optimized by Costa et al. (2000). *P. agglomerans* population dynamics on orange surface was monitoring using the method described by Teixidó et al. (2001).

**Laboratory studies to test different additives in order to improve *P. agglomerans* adherence and persistence**

In laboratory studies, different additives, such as summer oils, alginate, glycerol and food additives at two different concentrations were tested mixed with *P. agglomerans* in order to study its compatibility. The non-toxic ones (Citroline 0.2%, Summer oil 0.5%, Alginate 0.1%, Sunspray 0.2%, Glycerol 5%, Stapiot 0.1% Food Coat 3.3%) were added to the biocontrol agent, sprayed on detached oranges and left outdoors. Population dynamics of the antagonist on fruit surface was determined along the time. Different concentrations (2, 3.3 and 5%) of the best additive (FC) were tested in order to find the optimal dose to apply at field trials. Fungicide effect against *P. digitatum* and *P. italicum* was studied on oranges.

**Pre-harvest treatments of *P. agglomerans***

Oranges cv. Valencia Late and Lane Late were used in all experiments. Fruit trees, and fruits were from commercial orchards from two Baix Ebre-Montsià areas, Catalonia, Spain, and were grown under standard cultural practices. The sample unit used in field trials was five trees and all treatments were repeated four times. In all cases guard trees were used to separate randomized treatments and replicates. The experiment was repeated twice.

Pre-harvest treatments at field conditions with the biocontrol agent (2×10⁸ cfu ml⁻¹) alone or in combination with FC were sprayed in oranges 7 days before harvest simulating commercial treatments. Population dynamics on orange surface were monitored at 0, 2 and 7 days as described above.

**Efficacy from pre-harvest treatments to control post-harvest diseases**

Two boxes of fruits per replication were picked up from both treatments (BCA alone and BCA+FC) and 6 boxes per replication from control (non pre-harvest treated fruits) in order to test efficacy against post-harvest diseases. The treatments assayed were: Non-treated control: Fruits directly from field without any treatment to control post-harvest diseases. (2 boxes × 4 replications) CONTROL; Chemical treatment: Non pre-harvest treated fruits immersed during 30 seconds in a bath with 7.5 l Imazalil/100 l water (commercial fungicide and dose). (2 boxes × 4 replications) CHEMICAL; Fruits treated with *P. agglomerans* alone at pre-harvest (2 boxes × 4 replications) BCA PRE; Fruits treated with *P. agglomerans* in combination with FC at pre-harvest (2 boxes × 4 replications) (BCA+FC) PRE; *P. agglomerans* post-harvest treatment: Non pre-harvest treated fruits immersed during 60 seconds in a bath with 1×10⁸ cfu ml⁻¹ of *P. agglomerans* (recommended post-harvest concentration). (2 boxes × 4 replications) BCA POS. The assay was carried out per duplicated with natural infection and with artificial inoculation with *P. digitatum* sprayed at 1×10⁵ conidia ml⁻¹ after the mentioned treatments. Once dried outdoors, fruits were incubated at 20ºC and 85% RH. Percentages of decayed fruits were determined 7 and 15 days after incubation. Population dynamics on orange surface was also determined during this period.

**Results and discussion**

**Laboratory studies to test different additives in order to improve *P. agglomerans* adherence and persistence**

The additive that provided the highest bacterial viability on oranges was FC at 5% both just after treatment (best distribution on orange surface) and after 72 h outdoors (best persistence). This product is an edible food protector film for fruits and vegetables from DOMCA S.A. to reduce weight loss, delay senescence, improve natural brightness and reduce physiological disorders.
FC improved treatment homogeneity because it decreases droplet size and forms a uniform film on the fruit surface, in contrast with the other treatments in which big drops could be observed on sprayed oranges. The product does not have any fungicide effect against the studied post-harvest pathogens: *P. digitatum* and *P. italicum*.

**Pre-harvest treatments of *P. agglomerans* with or without FC addition (Season 2005-06)**

(BCA+FC) PRE treatment showed higher viability than BCA PRE during all the study, just after treatment (once oranges dried), along the time at field conditions and during post-harvest incubation (Fig. 1). Both pre-harvest treatments were sprayed at the same concentration, however, just after drying fruits (approx. 2 hours after treatment) initial population with (BCA+FC) PRE treatment was more than 2-log higher than BCA PRE. This difference was maintained until harvest. In post-harvest, the population of BCA PRE after 7 days was lower than 10 cfu ml⁻¹, meanwhile the population on FC treated fruits was already >10³ cfu ml⁻¹. Bacterial population on fruits treated at post-harvest was a little bit higher, about 10⁴ cfu ml⁻¹.

![Log (CFU/cm²) vs Time (days) with Harvest](image)

**Figure 1.** *P. agglomerans* population dynamics on orange surface in the different treatments assayed: the BCA treated alone at pre-harvest (BCA PRE ▲), the BCA treated in combination with 5% FC ((BCA+FC) PRE ■) and the BCA treated at post-harvest (BCA POST ◆). Pre-harvest populations (0, 2 and 7 days) and post-harvest populations at 20°C and 85%RH (15 and 21 days) are shown. Vertical bars indicate standard deviations.

**Efficacy from pre-harvest treatments to control post-harvest diseases**

Efficacy data were remarkable and percentages of rot reduction in relation to control prove in natural and artificial inoculation trials were around 40, 60 and 70% with BCA PRE, (BCA+FC) PRE and BCA POST, respectively. Results are similar in both conducted efficacy trials (Lane Late and Valencia Late), although, in general, the second one had lower percentages of decayed fruits. Fig. 2 shows an example of these results in artificial inoculation trials. The additive FC improved the efficacy of *P. agglomerans* and the results obtained with pre-harvest treatment with this additive were statistically similar to the post-harvest ones.

It is very important to optimize both, distribution of the biological control agent on the host surface and application techniques. Spray deposition, droplet size, spreadability and evaporation rate can be improved by the addition of surfactants, film-forming compounds, thickeners and humectants. FC is an edible film-forming compound, which reduces droplet size and improves uniformity of distribution on the surface to be protected. The main conclusions of this study are that it is possible to control post-harvest diseases with pre-harvest antagonist treatments and Food Coat additive improves the antagonist survival and efficacy.
Figure 2. *P. digitatum* fruit rot incidence after 7 (■) and 15 (□) days of incubation at 20°C and 85% relative humidity from the pre-harvest assay treatments conducted during season 2005-06 with Lane Late oranges. Artificial inoculation with *P. digitatum* was carried out on post-harvest. Columns with different letters indicate significative differences (*P*<0.05).

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References


Application of beneficial microorganisms to seed during priming to improve crop health and establishment

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Abstract: Specific fungal (Clonostachys rosea and Trichoderma harzianum) and bacterial (Pseudomonas chlororaphis and P. fluorescens) isolates were successfully applied to carrot and onion seed during drum priming, achieving a target rate of at least $5 \log_{10}$ cfu g$^{-1}$ dry seed. Subsequent glasshouse experiments in three soil types showed that priming seed with selected beneficial microorganisms could improve seedling emergence in some instances. Survival studies also showed that the microorganisms applied to seed during priming could be recovered from seedling roots and rhizosphere soil up to eight weeks after planting. The ability to deliver beneficial microorganisms successfully to seed during priming has potential to improve crop health and establishment.

Key words: disease control, microbial ecology, plant growth promotion, rhizosphere competence

Introduction

Seed priming is a process that helps to speed up germination, and improves seedling establishment in many crop and ornamental plants, especially under unfavourable soil conditions. Drum priming in particular is a process that can be used on a commercial scale. Previous work has shown that indigenous microorganisms increase in number during the drum priming process (Wright et al., 2003a), and that certain beneficial microorganisms applied to seed during drum priming can survive and proliferate on the seed (Wright et al., 2003b). There is considerable interest in the application of beneficial microorganisms to seeds to promote seedling establishment and growth, and control disease.

In the current study, microorganisms were applied to carrot and onion seed during priming, and glasshouse bioassays were conducted to assess whether seed primed with microorganisms showed improved emergence and establishment. Further studies also determined the continued survival of the seed-applied microorganisms on seedling roots and in the rhizosphere.

Materials and methods

Fungal and bacterial isolates
Fungal (Clonostachys rosea and Trichoderma harzianum) and bacterial (Pseudomonas chlororaphis and P. fluorescens) isolates were selected based on their availability within commercial microbial inoculants or for their known biocontrol or plant growth promoting properties. For application to seed, fungi were grown on potato dextrose agar and, when profuse sporulation had occurred, plates were flooded with sterile distilled water (SDW) and the spores scraped into suspension. The suspension was filtered through sterile lens tissue and haemacytometer counts estimated the number of colony forming units (cfu) present. Bacteria
were grown in nutrient broth culture, with numbers of cfu estimated by spectrophotometry after 2-3 hours incubation at 26°C in a rotary incubator (180 rpm). Samples of this batch culture were centrifuged (5000 rpm, 10 mins) and the bacteria were resuspended in SDW for addition to the seed. For both bacterial and fungal isolates the target application dose was 5 log10 cfu g−1 dry seed.

**Seed priming**

Carrot and onion seed were primed using the drum priming system (Rowse, 1996). Priming consists of 3 main stages: i) **hydration**, where a specific amount of water is added to a seed batch over a set period, based on previous calibration; ii) **incubation**, where the hydrated seeds are kept in a rotating jar for 7 days, with air exchange through a humid environment; and iii) **drying back**, where the seeds are dried back to a low moisture content for storage. In this work, microorganism suspensions were added to the water used to prime the seed during the hydration process and seed was sampled after drying back to determine the successful survival of the applied microorganism on the seed.

**Glasshouse experiments – establishment of seedlings**

Fully replicated glasshouse bioassays were set up in three soils (sandy clay loam, light sandy loam and peat) to assess the emergence and growth of carrot and onion seeds primed with the selected microorganisms. Emergence was assessed daily until no further increase in numbers was seen, and after 8 weeks the seedlings were harvested to determine the fresh weight.

**Glasshouse experiments – survival of microorganisms**

Fully replicated glasshouse bioassays were also set up in three soils (as above) to determine the survival of microorganisms on seedling roots and in rhizosphere soil following application to seed during priming. For these experiments antibiotic resistant strains of the microorganisms were used to allow for ease of identification from soil and root samples. Enough pots were set up initially to allow for destructive harvesting at 2, 4 and 8 weeks post planting. At each sample time loose soil was shaken off the harvested seedling roots and the closely adhering rhizosphere soil was then washed off in SDW. A dilution series of this soil suspension was made and plated onto selective media for isolation of the applied microorganisms. The roots were then blotted dry and ground using a sterile mortar and pestle. A suspension of the macerated root tissue was made in SDW and plated similarly.

**Results and discussion**

**Seed priming**

All microorganisms were successfully applied to carrot and onion seed and achieved final numbers above the target rate of 5 log10 cfu g−1 dry seed after the drying back process.

**Glasshouse experiments – establishment of seedlings**

For the carrot seed, all primed treatments emerged significantly faster than the unprimed control (Fig. 1a). Seed primed with *C. rosea* also had a significantly faster mean emergence time than the primed control, although there was no significant difference in the final percent emergence.

In the onion experiment the primed control unexpectedly had the worst emergence of all the treatments (Fig. 1b). This was probably due to a background population of deleterious microorganisms present on the seed batch, which increased in number during the priming process and had a negative effect on the onion seed emergence. However, the addition of beneficial microorganisms to the seed batch improved emergence of the primed seed compared to the control. Here, seed primed with *T. harzianum*, *P. chlororaphis* or *P. fluorescens* emerged significantly faster than the primed control (Fig. 1b), illustrating the
potential for the application of beneficial microorganisms to improve emergence of contaminated seed batches. The application of beneficial microorganisms may also have potential for disease control as beneficial microorganisms may directly parasitize pathogens, outcompete them for space or nutrients, produce antibiotics or inhibitory enzymes, or induce resistance in the plant (Whipps, 1997).

**Figure 1: Emergence of carrot and onion seed primed with different microorganisms**

**Glasshouse experiments – survival of microorganisms**

Similar trends were found in three soil types for the survival of applied microorganisms on carrot and onion following application during priming. Consequently, a representative example of carrot from one soil type only (peat) is illustrated in Fig. 2. In this soil, *P. fluorescens* declined slightly in number, but was still recoverable at >2 log₁₀ cfu per seedling after 8 weeks (Fig. 2a). In contrast, *P. chlororaphis* did not survive well, with numbers generally <1 log₁₀ cfu per seedling at all sampling times (Fig. 2b). *Clonostachys rosea* increased in number on the root and in the rhizosphere soil, with numbers reaching nearly 4 log₁₀ cfu per seedling at the end of the experiment (Fig. 2c). *Trichoderma harzianum* also increased in numbers, to a final figure of approximately 3 log₁₀ cfu per seedling by 8 weeks (Fig. 2d).
Figure 2. Survival of microorganisms on carrot roots and in rhizosphere soil after application to seed during priming. Seeds were planted in a peat soil (see also Figure 2 d next page)
In these experiments plants were watered from below, so that any applied microorganism recovered from the roots was due to growth of the fungus or bacterium itself, rather than the cells or spores being washed down from above. In order to improve crop growth and establishment, it is important that beneficial microorganisms specifically applied to the seed during priming can subsequently become established in the root zone of the plant. Such rhizosphere competence was shown in this work, particularly with the fungal isolates.

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**References**

Information, opinions and future perspectives on biocontrol agents among growers: comparison between two countries

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Abstract: Biocontrol agents (BCAs) are promising tools for pest control in agriculture, thanks to their low environmental impact and absence of chemical residues in the final product. The use of BCAs in pest control of strawberry could be a valid alternative to chemical pesticides, providing healthier produce for consumers and less impact to the environment. Growers’ viewpoint on BCAs can help researchers in identifying weak points and strategies to promote BCAs use in agriculture. A survey, aimed to understand how BCAs are considered and used by strawberry growers and what aspects could be implemented to increase BCAs utilization, was done in two different agricultural systems, i.e. northern Italy and Israel. The two selected areas differ in their climate, environment, farm dimension, market and growers organization: northern Italy (Trentino) has a continental climate, is a mountain area, farms are numerous, small and organised in cooperatives, mainly producing for the national market during summer; Israel (central part) is flat with Mediterranean climate, farms are bigger and focused on export in winter. Differences involve also phytosanitary problems, product efficacy and hence grower’s satisfaction. Representative samples of growers were selected and interviewed. Similarities and differences in information level and opinions between strawberry growers of the two countries are presented. Result show also that marketing and experience have a key role to widespread the practice of BCAs among the growers.

Key words: alternative methods, consumer, health, increase, strategy

Introduction

Biocontrol agents (BCAs) are living microorganisms naturally existing in the environment, used to control a parasite population within a threshold, so its density does not cause economical damages to the crop (Chalet, 2002). Thanks to their low environmental impact and to the absence of chemical residues in the final product, the BCAs are one of the most promising tools in plant protection (Pritts, 2000), with remarkable benefits for consumers, growers and the environment. BCAs are not dangerous to human health, because they are chosen among the non toxic microorganisms. They can therefore contribute to reduce the risk of acute or chronic poisonings for the workers who apply treatments in fields. They are supposed to have a minor impact on the environment, the fertility of the land and the aquifers (Hokkanen & Lynch, 2003). Considering the perspectives of the development of a new BCA, the attention of the researchers is mainly focusing on the efficacy against pests and diseases and on their mechanism of action. A crucial aspect, often neglected in the research of new agents, is the economic appraisal of their introduction in integrated pest management schemes and the social-economic obstacles that may prevent a wider use of the BCAs in practice.
In 2004 and 2006 an empirical survey was conducted on the growers’ perception of BCAs use in agriculture. Our goal was to understand the most relevant aspects for improving dissemination of BCA based techniques. The aims of the survey were to investigate the level of knowledge on BCAs, to understand what can influence the confidence in BCAs, to identify the major problems encountered with the use of them, to individuate strategies to facilitate BCAs introduction in field practices.

Material and methods

The survey
Strawberry production was selected and we interviewed growers, technicians and managers in two different agricultural systems: in Northern Italy (Trentino) and Israel. The two countries represent a “mature” area (Trentino), where IPM was introduced more than 25 years ago, and an area of relatively recent launch (Israel), where IPM it is applied since less than ten years.

A qualitative survey, using a semi-structured questionnaire was chosen and designed according Silverman (2000) and Corbetta (1999). Having given the nominal nature of most variables, the characteristic of the questions and because of the small dimension of the sample, data were statistically analysed using mode, frequency count and the relative frequency distribution. The final form of questionnaire was achieved throughout several subsequent drafts. In designing the first draft, we performed a qualitative preliminary evaluation of the most informative questions. Then the questionnaire was detailed designed according the principles of market research (Corbetta, 1999) and the psychological criteria for experimental design (Converse & Presser, 1986). The final version of questionnaire was formed after several internal and external reviews. The questionnaires were filled through personal interviews. The time allocated for each interview was about 20-30 minutes. Participants were guaranteed anonymity and the information reported has been reviewed, so neither individual person nor company can be identified.

The populations
The survey involved strawberry growers of Trentino Region (Italy) and Sharon Area (Israel). The two areas differ in climate, environment, farm dimension, market, and growers organization. Trentino Region is a mountain area having a continental-alpine climate with cold and snowy winters, cool summers, rainy springs and autumns. Farms involved in berry production are numerous (1500 with 130 of which are exclusively producing strawberry), with small surface (50% farms have less than 0.3 ha, 34% have 0.5-1.5 ha and 16% have 3-4 ha). The total strawberry surface is almost 60 hectares and it represents 1.1% of the Italian strawberry production surface. Growers are organised in cooperatives, and they produce mainly during summer for the national market (90%). Sharon Area in Israel is flat, has a Mediterranean climate characterized by hot and dry summers (with temperature over 30°C and almost zero precipitations) and cool rainy winters (daily average temperature of 12°C and 530 mm of rain). Farms are less numerous (180) and bigger compared to Trentino (67% farms have 2-5 ha, 28% have less than 2 ha and 5% have 5-25 ha) covering almost 400 ha and representing 95% of the Israeli strawberry production surface. They are also organized in cooperatives but are most focused on export (86%) especially during winter.

The sample
The survey was identified in order to broadly cover the cultivated area. In Trentino we selected 22 growers (13% have less than 0.5 ha, 40% have an area between 0.5 and 1.5 ha and 45% have big farm with more than 3 ha), representing approximately 40 ha out of the 60 ha. The average cultivated area of the farm sample is less than 2.5 ha. The average age of the interviewees is 41 year old, nearly all of them (86%) are males and half of them have a high
school diploma (55%). Most of them own the land (86%), work full time (72%) with the family (90%) and have worked in their farm for an average of 17 years (Moser, 2005).

In the Sharon area we selected 44 growers, 30% of which have less than 2 ha, 41% have an area between 2-5 ha and 29% cultivate an area between 5-25 ha, covering almost 180 ha. The average cultivated area per farm is bigger than in Trentino and corresponds to an average of about 4 ha. The age of the interviewees is 46 year old, all of them are males and most of them have a high school diploma (82%). Most of them own the land (88%), work full time (97%) usually with the help of a son (82%) and have worked in their farm for almost 19 years.

We also interview 17 managers and technicians in the two different countries (10 technical experts from extension services in Trentino and 7 managers in Israel) to obtain additional information and subjective opinions. We used these to comment the results.

**Results and discussion**

*Level of knowledge on BCAs*

The major BCAs features known by growers and common to the two samples are the “lower environmental impact”, the “higher cost, including the cost of treatment plus the cost for the monitoring”, “that BCAs give healthier products”, “are linked with higher safety for who is doing the treatment” and “more sensitive to climate factors” (Fig. 2). There are some relevant differences between the two groups. The most important is the feature “the chemical residues are absent (non detectable)”. While the Italian growers perceive the healthiness and safety of BCAs, they do not correlate them with the absence of chemical residues. Conversely the Israeli growers perceive it. This can partially explain why the consumer is not keen to pay higher price for the strawberry obtained with BCAs (in Italy). The growers in this case are acting as consumers. Moreover, we discovered that the Italian growers are more risk adverse than the Israeli ones, perceiving more risk even if it could not be present.

![Figure 1. Level of knowledge on BCAs in the two samples (percentage of the sample that answered positively).* Compared to chemical pesticides](image-url)
**Confidence in BCAs**

In the comparison between Israeli and Italian growers (Fig. 2), we can see that the former trust more BCAs (91%), than the latter ones (77%) and none of the Israeli does not trust at all, while some Italians do not trust them (9%). The factors that influence the confidence in using BCAs are, for both samples, the personal and positive experience and the suggestions made by the cooperative/growers association (Fig. 3). The big difference in confidence in BCAs between the two groups may be due to the role of advertising and project participation play in Israel. In Israel a lot of money and effort are put in advertising the benefits linked BCAs use. In this stage, in order to push the use of BCAs, it is also important that the growers that use with success talk and trust each other. Moreover a strong participation of growers in different demonstrative projects makes them more confident with the use and increase the trust.

![Figure 2. Trust in BCAs](image1)

![Figure 3. Factors that can influence the confidence in BCAs](image2)

**The main problems encountered in BCAs use**

Our findings show that the practical constraints encountered by growers are: the impossibility to reach a total control of the disease through the BCAs use, that they must intervene in the right time (BCAs can loose efficacy if they are applied too much in advance or too late), the BCAs high susceptibility to climate conditions, the time needed for monitoring the pest and the crop, the higher cost of BCAs compared to chemicals (Fig. 4).

![Figure 4. The main problems encountered with the use of BCAs](image3)

Even if these are common problems of the two groups, they have a different relevance in the two groups. The main problem for the Italian group is the relevance of time spent...
monitoring (27%), while for the Israeli is the lack of total control (45%) that is perceived as important. A relevant, but negative remark is that Trentino’s growers consider that the time spent in the filed to monitor the crop is seen as a waste of time. This can partially explain with the fact that in Trentino growers are mostly part-time workers in their farm and this additional activity reduces their free time. The Israeli group is more worried about achieving a satisfying control and the influence of wheatear. This can be explained with the fact that the farm is the sole source of income and the dry and warm climate can be prohibitive for the establishment of some BCAs. In both samples has to be noted the relevant number of farmers, who chose to not answer.

**Strategies to increase BCAs use**

To increase the use of BCAs, the managers and directors of the two groups follow different strategies. The differences in the socio-economical environment between the two countries are influencing both, the chosen strategies and the reactions they generated. In Israel the introduction of IPM is relatively young compare to Trentino. The chosen strategies have been giving surprising results. The use of BCAs (measured in number of ha of strawberry involved in the use) has increased from 2 to 210 ha during the period 1997-2003, which correspond in an increase from 0.5 to 67%. In Israel the successful application of IPM has reduced 30% of the use of insecticides, increased fruits consumption and farmers’ income (Gnayem et al., 2004). The Israeli cooperatives implemented several strategies to increase the use of BCAs, which were perceived by the farmers to different levels as being relevant: 1) to invest in advertising with the aim to differentiate the product obtained with BCAs on the market and at the same to make the consumers aware of the positive characteristics of IPM products (perceived by 71% as relevant); 2) to give financial help to the growers in order to cover the costs and the risks of changing from conventional to BCAs (57%); 3) to offer free assistance early in the program and increasingly charging the costs to them (42%). As consequence of combination of these three strategies they could increase the price for IPM products (43%) and therefore guarantee to the growers an equal profit (Fig. 5).

![Figure 5. Perceived relevance of the strategies to introduce or to increase the use of BCAs](image-url)

In Italy, almost all the production is following IPM rules, so the introduction of additional BCAs is not perceived by the consumers as a difference in the quality product therefore not rewarded financially. Moreover since the basic good agronomical practices and the beneficial are of wide used, the additional BCAs that can be introduced are mainly microbials that are know to be less effective than insects and mites. Therefore the Italian managers (Fig. 5) think that the most important strategies to promote the further application
of BCAs are the financial help (90%) that can cover the additional costs, followed by offering free assistance (50%).

Conclusions

Our survey analyzed the approach in using BCAs in two different situations. According to our findings, a wider use of BCAs could be possible following deep analysis the socio-economical environment in which they have to be applied, the crop and the market. Increasing efficacy and consistency of BCAs against pest and disease in field is obviously of crucial importance, but financial help (subsidies) and free assistance to growers represent a desired tool to achieve an increase of BCAs use. Demonstrations in experimental farms can be a good way to let growers touch with their hands the possible use of BCAs. Another important and crucial factor is directed to the consumers with the promotion of the strawberry obtained with the BCAs, by using the media like a spot in TV or a road poster. It is a crucial factor that can lead to an increase of market price for IPM strawberries and therefore to a warranty of a premium price for the growers that want to produce in a more sustainable way.

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References

Formulation of *Epicoccum nigrum* and *Penicillium frequentans* conidia to improve the biocontrol of post-harvest brown rot of peaches

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**Abstract:** Stabilizers were added to conidia of *Epicoccum nigrum* and *Penicillium frequentans* to improve shelf-life of conidia stored at different temperatures. Effect of additives on each fungal viability depended on the moment of their application. 1% KCl, 50% PEG 8000, 2.5% methyl cellulose, 2.5% methyl cellulose+ tale or 1% KCl + silica powder increased conidial viability of *E. nigrum* after 365 days of storage at room temperature, while 7.5% glucose, 7.5% glycerol, 1.5% sodium alginate or 7.5% sodium glutamate increased conidial viability of *P. frequentans*. A significant effect of storage temperature was observed on conidial viability of *P. frequentans* with or without additives after 365 days. No effect was observed with presence or absence of light or high vacuum. These formulations significantly reduced brown rot on peaches.

**Introduction**

*Epicoccum nigrum* Link and *Penicillium frequentans* Westling, both of them components of the resident mycoflora of peach twigs and flowers in Spain (Melgarejo et al., 1985) reduce peach twig blight and fruit rot caused by *Monilinia* spp. in field orchards (De Cal et al., 1990; Larena et al., 2005). Treatments in these trials were made with fresh conidia obtained from potato-dextrose agar (PDA) cultures, or from solid-state fermentation. Viability of these fresh conidia was quickly lost (De Cal et al., 2002; Larena et al., 2004) and conidial shelf-life was enhanced by drying conidia in a fluidized bed-dryer (Larena et al., 2003; Guijarro et al., 2006). Conidia dried by this method maintained 100% viability after 90 days of storage at room temperature, then decreasing to 30% or low after 180 days (Larena et al., 2003; Guijarro et al., 2006). The principal objective of this work was to find conidial *E. nigrum* and *P. frequentans* formulations that maintain a high viability and efficacy.

**Materials and methods**

**Cultures**
Conidia of *E. nigrum* (ATCC No 96794) and *P. frequentans* (ATCC No 66108) were produced in a solid fermentation system (De Cal et al., 2002; Larena et al. 2004) and dried in a fluid bed-dryer until moisture contents below 15% (Larena et al., 2003; Guijarro et al., 2006). *Monilinia laxa* (ATCC No 66106) conidia were produced on PDA.

**Additives**
Stabilizers or/and desiccants, such as sugars (sucrose, glucose, maltose, lactose, and d-sorbitol), polyalcohols (glycerol, PEG 300, PEG 8000), salts (NaCl, KCl, sodium glutamate), detergents (Tween 20, Tween 80, Triton TX100), antioxidants (ascorbic acid, peroxidase), hydrophobic compounds (sodium alginate, methylcellulose), desiccants (silica powder, CaCl₂, etc.)
kaolinite, talc) and other compounds (peptone, gelatine, dimethyl sulphoxide) were used at different doses. Additives were dissolved in distilled water w:v (solid compounds) or v:v (liquid compounds) and then autoclaved at 1.0 Kg cm$^{-2}$, 120ºC for 20 min.

**Shelf-life assays**
Additives with non-toxic effects on germination and/or production of *E. nigrum* and *P. frequentans* conidia were tested to improve conidial stability. To determine the effect of additives on viability of conidia, additives were added to conidia at three stages of the production-drying process: i) to substrate contained in bags before production, ii) to conidial centrifuge pellets obtained after production before filtering and drying, iii) to conidial centrifuge pellets obtained after production before adding talc and drying, and iv) to conidial centrifuge pellets obtained after production, before adding silica powder and drying. The highest non-toxic dose of each additive per fungi was used. *E. nigrum* and *P. frequentans* conidia without additives were used as controls. Three replicates were made per fungi, additive and step. Viability of *E. nigrum* and *P. frequentans* conidia were estimated by measuring their germination just after fluid bed-drying and at 30, 90, 180 and 365 days of storage at room temperature by the bioassay described in De Cal et al. (2002). All experiments were repeated twice. In the case of *P. frequentans* conidia with the different additives were also stored for 365 days in different conditions: 4ºC or room temperature (ambient), with or without light and at a high vacuum or without vacuum (three replicates for each combination). Viability of conidia was estimated by measuring their germination just after fluid-bed drying and after 365 days of storage by the bioassay mentioned above.

**Biocontrol efficacy of conidial formulations**
Three 1 mm$^3$ artificial wounds 2 cm apart were made on healthy surface sterilized peaches. Fifty ml of a conidial suspension of each *E. nigrum* or *P. frequentans* formulation (10$^6$ conidia ml$^{-1}$) was sprayed on the fruits. The following formulations were applied: FOR1) a conidial suspension of *E. nigrum* produced with 50% PEG8000, FOR2) a conidial suspension of *E. nigrum* produced with 1% KCl, FOR3) a conidial suspension of *E. nigrum* maintained with 2.5% methyl cellulose for 10 min before drying, FOR4) a conidial suspension of *E. nigrum* maintained with 1% KCl for 10 min, then pre-dried with silica, and then dried, FOR5) a conidial suspension of *P. frequentans* produced with 10% glycerol, FOR6) a conidial suspension of *P. frequentans* produced with 2% sodium alginate, FOR7) a conidial suspension of *P. frequentans* maintained with 10% glucose for 10 min before drying, FOR8) a conidial suspension of *P. frequentans* maintained with 10% sodium glutamate for 10 min before drying and FOR9) a conidial suspension of *P. frequentans* maintained with 2% sodium alginate for 10 min before drying. Twenty µl droplets of a conidial suspension of *M. laxa* (10$^4$ conidia ml$^{-1}$) were applied to the three artificial wounds. Fruits were incubated for 4-5 days at 20-25ºC under 80% RH in darkness. *E. nigrum* and *P. frequentans* conidia without additives were used as controls. Ten fruits were treated, with three wounds per fruit, and the complete experiment was repeated twice. Disease incidence (as percentage of rotten wounds) and diameter of each lesion (mm) caused by *M. laxa* in peaches were recorded at the end of the assay.

**Data Analysis**
Data were analysed by analysis of variance. When F-test was significant at $P = 0.05$, means were compared by Student-Newman-Keul’s multiple range test. Correlation and regression analyses were carried out with percentage of disease control and percentage of conidial viability.

**Results and discussion**
*E. nigrum* and *P. frequentans* showed a good potential for development as commercial biocontrol products against brown rot of peach fruit. Wettable-powder formulations of *E.
and P. frequentans conidia with a shelf-life of 365 days have been developed. A significantly higher conidial viability of E. nigrum was recorded after 365 days of storage at room temperature when 1% KCl (49% viability) or 50% PEG 8000 (44% viability) were added to substrate in bags before production (case i), when 2.5% methyl cellulose (48% viability) was added to conidial centrifuge pellets obtained after production before filtering and drying (case ii), and when 2.5% methyl cellulose + talc (case iii) (37% viability) or 1% KCl + silica powder (case iv) (72% viability) was added to conidial centrifuge pellets obtained after production, before adding dessicants and drying.

A significantly higher conidial viability of P. frequentans was recorded after 365 days of storage at room temperature when 7.5% glucose (31% viability), 7.5% glycerol (46.2% viability) or 1.5% sodium alginate (45.5% viability) were added to substrate in bags before fermentation (case i), when 7.5% glucose (44.9% viability), 7.5% sodium glutamate (38.5% viability), or 1.5% sodium alginate (38.4% viability) were added to conidia before drying by bed fluid drier (case ii). Conidial viability of P. frequentans without any additive (control) and stored at 4°C was significantly higher (38%) than that of conidia stored at room temperature. A significantly higher conidial viability was recorded after 365 days of storage at 4°C when 7.5% glucose (75.8% viability) or 1.5% sodium alginate (87.0% viability) were added to substrate in bags before fermentation, and when 7.5% glucose (69.7% viability), or 1.5% sodium alginate (47.7% viability) were added to conidia before drying. Storage temperature is the most important abiotic factor that affects the shelf-life of biological formulations by maintaining them in a state of low metabolic activity. No effect was observed on conidial viability after 365 days of storage by presence or absence of light or high vacuum.

Formulations of E. nigrum FOR1, FOR2 and FOR4 reduced disease incidence by more than 20%, and lesion size by 27, 57, and 64%, respectively (Fig. 1) despite the high pressure of disease (100%). However, FOR3 and E. nigrum conidia dried without additives reduced only lesion size (60 and 35% respectively). All formulations of P. frequentans reduced disease incidence by more than 50% while P. frequentans conidia dried without additives only reduced 25% disease incidence (Fig. 2).

Furthermore, shelf-lifes of E. nigrum and P. frequentans conidia were positively correlated with biocontrol efficacy ($r=0.74$ and $r=0.59$, respectively). Our results indicate that it is necessary to add additives to conidia produced in solid-state fermentation and dried afterwards for improving E. nigrum and P. frequentans shelf-lifes and for obtaining a highly effectiveness in controlling brown rot of stone fruits.

![Figure 1. Percentages of significant control of different formulations of E. nigrum on disease incidence and lesion size caused by M. laxa on peaches, and percentage of viability of each formulation](image-url)
Figure 2. Percentages of significant control of different formulations of \textit{P. frequentans} on disease incidence caused by \textit{M. laxa} on peaches, and percentage of viability of each formulation.

### Acknowledgements

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### References


Production of lipopeptide antibiotic iturin A by *Bacillus subtilis* using soybean curd residue in solid-state fermentation, and evaluation of the product as biocontrol agent

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**Abstract:** *Bacillus subtilis* RB14-CS, which suppresses the growth of various plant pathogens *in vitro* by producing the antibiotic iturin A, was cultured using soybean curd residue in solid-state fermentation (SSF). After 4 day incubation, iturin A production reached 3300 mg/kg wet substrate. By the statistical optimization of medium compositions, iturin A production was enhanced to 5500 mg/kg wet substrate. When the okara product cultured with RB14-CS was introduced into soil infested with *Rhizoctonia solani*, which is a causal agent of damping-off of tomato, the disease occurrence was significantly suppressed. As the okara cultured with RB14-CS exhibited functions of both plant disease suppression and nutritional effect on tomato seedlings, this product is expected to contribute to the recycling of the soybean curd residue.

**Key words:** antifungal, fertilizer effects, optimization

**Introduction**

*Bacillus subtilis*, a representative gram-positive soil bacterium, is attracted as a candidate of biological control agents of plant pathogens. *B. subtilis* RB14 isolated in our laboratory, exhibited broad suppressive abilities to plant pathogens by the producing of the lipopeptide antibiotics iturin A. The effectiveness of RB14 cultivated in submerged fermentation in plant tests was proved (Szczech & Shoda, 2004; Kita et al., 2005).

On the other hand, a large amount of organic solid waste has been produced from the agriculture and food industry. The soybean curd residue, okara, a by-product of tofu manufacture, is disposed in amount of 0.7 million tons in Japan annually; most of them is incinerated. As this residue is rich in nutrients and consists of insoluble fiber (Khare et al., 1996) and approximately 70% water, it can be used as a substrate in solid-state fermentation (SSF) (Ohno et al., 1995) and production of lipopeptide antibiotics was significantly higher than that in submerged fermentation.

In this study, one derivative of *B. subtilis* RB14 was cultivated in SSF using okara as a main solid substrate and a product that has a dual function of microbial pesticide and organic fertilizer was produced and the efficasy of the product was confirmed.

**Material and methods**

**Strains**

As a producer of antifungal iturin A, *Bacillus subtilis* RB14-CS was used. *Rhizoctonia solani* K-1, isolated at Kanagawa Horticultural Experiment Station (Kanagawa, Japan) (Asaka &
Shoda, 1996) was used as a fungal plant pathogen that causes a severe damping-off in many plants.

**Solid-state fermentation (SSF)**

Fifteen gram of okara was placed in a 100 mL conical flask and autoclaved twice at 120°C for 20 min at an interval of 8-12 h. After cooling to room temperature, the following sterile solutions were added to every 15 g of okara: 375 mg of glucose, 10 mg of KH₂PO₄, 37 mg of MgSO₄·7H₂O and 367 µL of deionized distilled water, for the supplementation of nutrients and adjustment of moisture content to approximately 79%. Then, 3 mL of a preculture of *B. subtilis* RB14-CS was added to every 15 g of okara and mixed with a stainless steel spatula. All flasks were incubated at 25°C.

**Statistical optimization**

A central composite design with five coded levels in duplicate was used to determine the optimum amounts of additional carbon and nitrogen sources for production of iturin A. Glucose and soybean meal were used as additional carbon and nitrogen sources, respectively, because they were found to be most effective in enhancement of iturin A production among tested in the preliminary assay. The quadratic model for predicting the optimal point was expressed according to the following equation:

\[
y = b_0 + \sum b_i x_i + \sum b_{ij} x_i x_j + \sum b_{ijk} x_i x_j x_k
\]

where \( y \) is the response variable, \( b \) the regression coefficients, and \( x \) the coded levels of the independent variable.

**Plant test**

The sterilized soil (150 g), a mixture of a low humic andosol and vermiculite, was placed in a plastic pot (90 mm diameter, 80 mm height), and its moisture content was kept at 60% of the maximum water-holding capacity by daily addition of sterilized water. For the infection of soil, the mycelial mats of *R. solani* that formed on the surface of PDP medium were homogenized in sterile water and inoculated into the soil at a ratio of 3.6 g-mat to one pot 6 days before planting the germinated tomato seeds.

The plant test was performed according to the following procedure: Tomato seeds were disinfected with 70% ethanol and then with 0.5% sodium hypochlorite. After rinsing with sterile water, the seeds were germinated on a 2% agar plate at 30°C for 2 days in the dark. Each pot was sown with nine germinated seeds 3 days after the okara culture of RB14-CS was introduced into soil and placed in a growth chamber at 30°C with 90% relative humidity under 16 h of light (about 8,000 lux). After two weeks, the percentage of diseased seedlings per pot was determined.

**Results and discussion**

**SSF of *B. subtilis* RB14-CS**

In SSF, *B. subtilis* RB14-CS grew well and maximum cell number reached \( 2 \times 10^{10} \) CFU/g wet substrate. The iturin A concentration reached approximately 3300 mg/kg wet substrate in 4 days which is approximately 10-fold higher than that in submerged fermentation.

**Optimization of SSF**

To enhance the iturin A productivity in SSF, statistical optimization of carbon and nitrogen sources using response surface methodology was carried out. From the results of the efficient experimental design, an equation represented in Fig. 1 was derived. The estimated maximum iturin A concentration was 5591 mg/kg wet substrate with 0.998 g of soybean meal and 1.83 g of glucose per 15 g wet okara. In the confirmatory experiment, iturin A concentration reached 5454 mg/kg wet substrate after 4 days of cultivation. This strong correlation between experimental and statistical results shows the validity of the response model.
Figure 1. Three-dimensional diagram of the calculated response surface
\[ F \text{ value} = 91.49; \ P \text{ value} < 0.0001; \ R^2 = 0.96. \]

**Suppressive effects of solid culture of RB14-CS**
Suppressive effects of solid culture products of RB14-CS on the plant disease were investigated in the pot test (Table 1). When the soil was infected by *R. solani* K-1, 84% of tomato seedlings were diseased. Ordinary solid culture reduced diseased ratio to 52%. Inoculation of solid culture derived from optimized condition as shown in Fig.1 lowered diseased ratio to 19%. The efficacy of SSF product was enhanced in the optimized product, indicating that the enhanced iturin A production was more effective to suppress the occurrence of the damping-off.

<table>
<thead>
<tr>
<th>Run</th>
<th>R. solani</th>
<th>Okara cultured with RB14-CS</th>
<th>Diseased ratio (%)</th>
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</thead>
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<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3 a</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>84 d</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>Basal</td>
<td>52 c</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>Optimum</td>
<td>19 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LSD ((P = 0.05))</td>
<td>14</td>
</tr>
</tbody>
</table>

Means (\(N=9\)) with different letters are significantly different \((P=0.05)\) according to Fisher’s protected least significant difference (LSD) analysis.
**Fertilizer effects of solid culture of RB14-CS**

When raw okara was introduced to soil, the shoot length and dry weight of each seedling of tomato was lower than the control by more than 15 and 25%, respectively. This indicates adverse effect of raw okara on the growth of tomato. On the other hand, the introduction of okara product by SSF increased the shoot length by more than 10% and the dry weight of the seedling by more than 25%. This proves that the cultivated okara with RB14-CS in SSF has a dual function as an organic fertilizer and a biological control agent.

**References**


Increasing stress tolerance, epiphytic fitness and efficacy of biocontrol bacterial strains by means of osmo-adaptation

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Abstract: Biocontrol bacterial strains suffer several stresses during production, conservation and after application to plant surfaces. A procedure for the pre-adaptation of bacteria by osmoadaptation during inoculum preparation was developed to increase cell survival during unfavorable conditions. The method consists of the combination of saline osmotic stress and osmolyte amendment to the growth medium. The experiments were performed with the biocontrol agent of post-harvest diseases Pantoea agglomerans EPS125 and the biocontrol agent of fire blight Pseudomonas fluorescens EPS62e. Under osmotic stress cells accumulated compatible solutes intra-cellularly but decreased significantly growth rate and cell yield. The amendment of the saline growth medium with glycine betaine restored growth rate and cell yield. The osmo-adapted cells showed a strong increase in tolerance to desiccation and thermal stress (100 to 1000 fold). Osmo-adaptation also increased by 100-fold cell survival on plant surfaces and efficiency of biocontrol under low relative humidity conditions in comparison to the non-osmo-adapted controls. The implications of the method for increasing the epiphytic fitness and the efficacy in other biological control agents of plant pathogens are discussed.

Key words: fire blight, glucosyl-glycerol, glycine betaine, heat shock, NAGGN, osmotic stress, phyllosphere survival, Pantoea agglomerans, Pseudomonas fluorescens, post-harvest diseases, trehalose

Introduction

Biological control of plant disease has been the focus of a considerable research, several bacteria and fungi have been isolated and some are developed commercially as bio-pesticides (Janisiewicz & Jeffers, 1997; Johnson & Stockwell, 1998). However, the efficiency of biocontrol is greatly dependent on environmental conditions. Under favorable conditions many biocontrol strains colonize host plant surfaces and efficiently exclude pathogens from infection sites. Unfortunately, antagonists that are effective under optimal conditions may fail to acquire the threshold populations needed for biological control, because of difficulties to colonize or survive on plant surfaces (Stockwell et al., 1998). Moreover, the development of biocontrol agents as microbial pesticides requires mass production and formulation procedures (e.g. dehydration) to get a stable product through time for storage and commercial delivery (Burgues, 1998). Losses of viability can be high during the process of dehydration due to cell damage by desiccation and thermal stress (Rhodes, 1993). Therefore, survival during formulation and in the plant surface during the subsequent period after application are critical points for the commercial development of biological control agents.

Microorganisms survive hyper-osmotic stress by means of intracellular accumulation of compatible solutes and membrane stabilization that counterbalance the external increase in osmotic pressure, and maintain cell turgor (Csonka & Hanson, 1991). The process of hyper-osmotic adaptation allows cells to tolerate adverse conditions such as drought or salinity,
freezing and high temperatures, and has been used in biocontrol agents to increase tolerance to desiccation and efficiency of control plant pathogens (Bonaterra et al., 2005; Teixidó et al., 1998).

The aim of the present work was to study the effect of osmoadaptation of *P. fluorescens* EPS62e and of *P. agglomerans* EPS125 during inoculum preparation, on tolerance to desiccation and thermal stress, as well as on the colonization and survival capacity in different plant surfaces under low relative humidity conditions, and on the efficacy of biocontrol.

**Material and methods**

*P. agglomerans* EPS125 and *P. fluorescens* EPS62e were grown in a glucose minimal medium (GMM). The medium was supplemented with 0.1, 0.3, 0.5 or 0.7 M of NaCl. Glycine-betaine (GB) or trehalose (T) were also added in some experiments at 0.1 or 1 mM. For inoculation, a preculture was first prepared with the same medium alone or amended with NaCl, GB or T. Growth was monitored using a Bioscreen C microplate reader (Labsystems) by means of measurements of OD at 600 nm.

To determine intracellular osmolyte contents, bacteria were grown in GMM supplemented with NaCl and GB. Cells were harvested by centrifugation and the pellet was extracted with ethanol 70%. The ethanol extracts were rotary evaporated to dryness for analysis. NMR Spectra were obtained using a Bruker Avance-360 spectrometer and D2O as solvent. For the high performance liquid chromatography (HPLC) analysis the dry material was re-dissolved in acetonitrile-water (1/1, v/v) before analysis. Osmolytes were quantified by HPLC in a Waters HPLC (model 610, Waters, Milford, Madison, USA). The intracellular osmolyte contents were determined by comparison of peak areas with those of pure standard compounds (GB, T) or by a combination of HPLC areas and 1H-NMR peak integrals (*N*-acetylglutaminylglutamine amide (NAGGN) and glucosyl-glycerol (GG)), and expressed as specific contents in fg cfu⁻¹.

The effect of osmo-adaptation on survival to desiccation and thermal stress was studied. Osmoadapted cells were obtained by growth in GMM amended with NaCl alone (S) or plus GB (S-GB). Non-osmo-adapted cells were cultured in un-amended GMM. For the study of survival to desiccation culture aliquots of 1 ml were distributed into 1.5 ml Eppendorf tubes harvested by centrifugation and the pellet containing cells was dried in a vacuum desiccator using silica gel as desiccant. The tubes were maintained in the desiccator at 4ºC. Samples were taken for cell viability analysis at different times during a period of 40 days. For the study of tolerance to thermal stress, cells obtained from stationary phase cultures were transferred to a water bath maintained at 45ºC. Cell viability was assessed and data were transformed to log10 percentage of surviving cells.

The effect of the osmo-adaptation of EPS62e on the epiphytic fitness and on the efficacy of biocontrol was studied on flowers of pear (cv. Conference) and apple (cv. Fuji) and in immature pear fruits (cv. Williams and Conference). Flowers and immature fruits were spray inoculated with the bacteria suspension at 10⁸ cfu/ml (osmoadapted(S-GB) or non osmo-adapted (GMM). Weather conditions in the field plots were measured with a meteorological station. Sample for monitoring population levels were homogenized, serially diluted and seeded onto LB agar plates supplemented with 50 µg ml⁻¹ of nalidixic acid to counter-select the strain inoculated. The population levels of EPS62e were expressed as log₁₀ CFU per blossom or fruit. Open blossoms and immature fruits were also collected from field plots to perform efficacy assays under controlled environment conditions. The individual flowers for the susceptibility to *Erwinia amylovora* were prepared as described (Pusey, 1997). Immature fruits were wounded with a flame sterilized cork borer (four wounds per fruit) (5 mm depth, 3
mm diameter) and were placed in polystyrene tray packs which were introduced into plastic boxes and maintained overnight at 20°C and high relative humidity to permit wound colonization by the surface population of EPS62e. Then, the hypanthium of flowers and each wound of fruits were inoculated with a suspension of *E. amylovora* at $10^7$ CFU/ml. The inoculated plant material was placed in plastic boxes, and incubated at 21°C and high relative humidity for 7 days. Severity on flowers and fruits was evaluated per each replicate after 7 days of pathogen inoculation.

The effect of the osmo-adaptation of EPS125 on the epiphytic fitness and on the efficacy of biocontrol was studied on apple fruits (cv. Golden). Apple fruits were inoculated by immersion in a bacteria suspension at $10^8$ cfu/ml (osmo-adapted (S-GB) or non osmo-adapted (GMM)) and maintained at 20°C under three different conditions of incubation: high (90% RH), low (40% RH) and fluctuating (24 h low, 24 high RH) relative humidity. After 15 days of treatment, population levels of EPS125 on fruit surface were assessed, and the fruits were wounded with a flame sterilized cork borer (nine wounds per fruit) (3 mm depth, 5 mm diameter) and were incubated again at 20°C for 24 h. Then each wound was inoculated with a suspension of *Penicillium expansum* at $10^4$ conidia/ml and incubated at the same temperature. The lesion diameter was assessed at 3 and 7 days after pathogen inoculation.

**Results and discussion**

The growth of *P. agglomerans* EPS125 and *P. fluorescens* EPS62e was significantly affected by NaCl and osmolyte treatments. Growth was slight or absent in GMM amended with high concentrations of NaCl in comparison to the non-amended control. In all experiments performed, the addition of the osmo-protectant glycine-betaine restored growth rate and OD values to levels not significantly different than the control. However, the effect of trehalose was less important.

The osmolytes T, NAGGN and GG accumulated intra-cellularly in response to salt stress in *P. fluorescens* EPS62e cells whereas in *P. agglomerans* EPS125 cells only T was intra-cellularly accumulated. Cells cultured in non-NaCl amended media did not accumulate significant amounts of compatible solutes. The osmolyte cell contents increased upon increasing NaCl concentration. The maximum amount of osmolytes was observed in salt stressed cultures without amendment of the growth medium with GB. The addition of GB to the growth medium partially inhibited the synthesis of T, NAGGN and GG. GB was only detected intra-cellularly at significant levels when it was supplied externally to the saline growth medium.

Upon desiccation cell survival of EPS125 and EPS62e decreased through time, and for all treatments performed (non-treated, NT; salt stressed, S; salt stressed plus GB, S-GB). A significantly higher survival was observed in the S treated cells than in NT control during the first 10 days. However, at longer times (35 to 40 days) there were no significant differences in survival between S and S-GB treated cells.

Upon thermal treatment at 45°C the death rate in osmo-adapted cells was considerably reduced 2 to 4-fold compared to non-osmoadapted cells. A 100 to 1000-fold increase in cell survival was observed upon 40 min treatment at 45°C in S or S-GB treatments compared to the NT controls.

Cell survival on plant surfaces was dependent on the osmo-adaptation treatment during cultivation and the environment conditions. Survival of EPS125 on fruit surface and of EPS62e on apple leaf surface and immature fruit surface was higher at high RH than at low RH conditions. At low RH, NT cells survived less than S and S-GB treated cells, and differences between S and S-GB treatments were not significant, in all experiments
performed. The increase in cell survival in the S treatment in relation to the NT control was around 10 to 100 times. At high RH no significant differences were observed between treatments for most of the time.

Osmo-adaptation of EPS125 significantly improved blue mould control under conditions where the standard biological control treatments were ineffective. The rot diameter was significantly reduced in apple fruits treated with EPS125 and incubated for several days under low RH, followed by wounding and inoculation of *P. expansum*. The efficacy of control of fire blight infections on immature fruits treated in the field with EPS62e and inoculated with the pathogen in controlled environmental conditions was also significantly increased upon osmoadaptation but was less improved in blossoms when weather conditions were wet. It is expected that the procedure can be effective to optimize control efficacy with other biocontrol agents of fire blight or with other biocontrol agents of plant diseases, and may contribute to a successful commercial exploitation of microbial pesticides.

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Alginate matrix based formulation for storing and release of biocontrol agents

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Abstract: Fungi belonging to the genus Trichoderma are naturally present in the environment. Some of them are known to be biocontrol agents of foliar and root plant pathogens. One of the main constraints of microbial biocontrol agents is their short survival during storage and after field application, with consequent lost of efficacy. The objective of the present study was to explore the use of encapsulation with sodium alginate matrix of Trichoderma spp. as a new formulation with the aim to protect the microorganism from environmental stress (temperature, desiccation, contamination, etc.). Encapsulation has been suggested as an alternative method for entrapment and immobilization of whole cells or their extracts, but currently there are a limited numbers of reports describing the encapsulation of microbial cells. Alginites are widely used in food and pharmaceutical industries and have been utilised as a matrix to trap drugs, macromolecules and biological cells in controlled environment like bioreactors, but they are not used with microbial biocontrol agents.

Key words: BCAs

Introduction

Trichoderma spp. are fungi that are present in substantial numbers in nearly all agricultural soils and in other environments such as decaying wood. Many have parasitism ability, visible through the typically growth toward hyphae of other fungi, coiling around them in a lectin-mediated reaction, and degradation of cell walls of the target fungi. In addition to, or sometimes in conjunction with mycoparasitism, individual strains may produce antibiotics. These processes (mycoparasitism and antibiosis) can be exploited to limit growth and activity of plant pathogenic fungi. The antifungal abilities of these beneficial microbes have been known since the 1930s, and there have been extensive efforts to use them for plant disease control since then.

However, biocontrol agents (BCAs) are generally not sufficiently efficient to control the target to a similar extent than chemical fungicides. This is partially due to their environmental stress sensitivity which rapidly reduces the survival, for example temperature range, desiccation and UV radiation, and contamination/displacement by other fungi. To increase persistence in soil (survival, viability) of Trichoderma and ultimately its efficacy as a BCA we developed a protection system based on an alginate matrix.

The alginites are a family of natural polysaccharide found in brown algae, they are a linear 1,4 linked copolymers of β-D-mannuronic acid (M) and α-L-glucuronic acid (G). These monomers are arranged as blocks in a chain. The homo-polymeric regions of M blocks and G blocks are inter-dispersed with regions of alternating structure (MG blocks). Alginites have the ability to bind multivalent cations being the basis of their gelling properties, leading to the formation of covalent bonds yielding insoluble hydro-gels. This anionic polysaccharide forms strong gels with divalent cations like Ca^{2+}, giving both strength and flexibility. During the
process of gelification the soluble sodium alginate is cross linked with calcium chloride resulting in the formation of the insoluble calcium alginate.

**Materials and methods**

![Figure 1. Gelification of alginate polymers by adding Ca^{2+} ions](image)

Entrapment within calcium alginate is the most practical technique for immobilising cells. It is especially suited to living cells as it requires only very mild conditions. Applications of this versatile method include immobilisation of living or dead cells in bioreactors, entrapment of plant protoplasts and plant embryos ('artificial seeds') for micro-propagation, immobilisation of hybridoma cells for the production of monoclonal antibodies, and the entrapment of enzymes and drugs.

The cells or enzymes to be entrapped are first mixed with a solution of sodium alginate. This is then dripped into a solution containing multivalent cations (usually Ca^{2+}). The droplets form spheres automatically as they immerge, entrapping the cells in a three-dimensional lattice of ionically cross-linked alginate (Fig. 1).

The process to obtain entrapped *Trichoderma* conidia can be divided into two steps: first the mass production of *Trichoderma* conidia by fermentation in a solid state substrate. *Trichoderma* is inoculated onto the sterile substrate; harvest is made once substrate is completely covered by green *Trichoderma* conidia by rinsing with sterile water and collecting the conidia through centrifugation. The second step is conidia encapsulation with alginate matrix. As Sodium alginate is not readily soluble in water, it is first auto cleaved in bi-distilled or de-ionised water at 120°C. The conidia suspended at an appropriate concentration are now mixed with an equal volume of 4% alginate solution in a beaker and added drop wise (using a burette) to a continuously stirred 2% Calcium chloride solution. The resulting spherules with immobilised conidia inside are then left to harden 30 minutes in the calcium chloride solution. Afterwards they are collected on filter and rinsed with tap water and stored in a closed container. Survival of the conidia was assayed at different temperature (-18, +4, +25 and +45°C) and exposure time (1, 7 and 30 days). After each time the alginate spherules mass was placed in 0.2 M EDTA solution that seize Ca^{2+} ions so that the alginate matrix dissolves and releases the conidia in solution; which is used, after a serial dilution, for a plate count. The value obtained with plate count related with the initial amount of spore in solution give the survival percentage.
Results and discussion

Our *Trichoderma* formulation was compared with non formulated *Trichoderma* conidia and with the formulated commercial strain *T. harzianum* (Koppert T-22). Through the alginate encapsulating the proportion of surviving spores could be relevantly incremented compared to the non encapsulated (Table 1). *T. atroviride* 122F has a slightly larger range of temperature than the commercial *T. harzianum* T-22) as its survival is less reduced at the extreme temperatures.

Table 1. Alginate encapsulated conidia of *Trichoderma* in different condition and temperature compared with non encapsulated spores and with a commercial formulation

<table>
<thead>
<tr>
<th>Experimental conditions (°C)</th>
<th>Strain</th>
<th>Exposure time (days)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>-18</td>
<td>122F formulated</td>
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</table>

References

Survival in the phylloplane of *Trichoderma koningii* and biocontrol activity against tomato foliar pathogens

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**Abstract:** The biological control of tomato foliar diseases by a formulated prototype based on *Trichoderma koningii* (Th003) was evaluated under greenhouse conditions in comparison with the unformulated fungus. Survival of the antagonist on tomato phylloplane was studied. Although highest inoculum pressure of *Oidium lycopersicum* was present during the experiment, both formulated and unformulated *T. koningii* treatments reduced powdery mildew incidence by 25 and 28%, respectively, whereas disease severity was reduced from 28-66%. The study on population dynamics of *T. koningii* on tomato phylloplane showed differences in the viable propagules density between formulated and unformulated conidia.

**Keywords:** foliar pathogens, phylloplane, tomato

**Introduction**

Foliar diseases are very common and economically important in tomato greenhouse crops. Chemical application is the major means by which these diseases are controlled. The use of biological control agents (BCA) is of great interest due to the undesirable side effects of pesticides. *T. koningii* strain Th003 has shown to be effective in control of both soil-borne (Cotes et al., 2001) and foliar (Moreno, 2003) pathogens. A bio-pesticide prototype formulated as wettable powder based on Th003 recommended for foliar application was developed (Villamizar et al., 2004). In order to optimize the effectiveness of the BCA an adequate understanding of environmental and host factors, that influence the density dynamics of the antagonist and the development of the agent in the crop and its biocontrol activity, should be determined (Yu & Sutton, 1999). The aim of the present work was to study the survival of *T. koningii* on tomato phylloplane and to evaluate its efficacy in the foliar diseases control.

**Materials and methods**

**Plant material**
A total of 480 plug-raised tomato (*Lycopersicon esculentum* cv. Rocio, Syngenta Seeds INC.) plants were grown in a clay sand soil in an unheated greenhouse in five rows spaced 1.2 m from centre to centre and plants within rows with stems spaced 0.5 m apart. The plants were irrigated by a drip irrigation system and fertilized with N (13%) – P (26%) – K (6%) fertilizer and compost (2.5 Ton/Ha). No chemical pesticides and foliar fertilizers were used.

**Density patterns of *T. koningii* (Th003) on tomato phylloplane**
The density of colony forming units of *T. koningii* on tomato phylloplane (Log$_{10}$ CFU/cm$^2$) was quantified as a function of time. Treatments consisted of i) formulated product and ii)
unformulated conidia of *T. koningii*. The product was used according to recommendations, while the unformulated conidia were suspended in Tween 80 (0.01% v/v). In both cases the concentration of *T. koningii* was $1 \times 10^6$ conidia mL$^{-1}$. The suspensions were prepared approximately 3 hours before application by using a backpack sprayer. The experimental unit consisted of 24 plants. The experimental design was a randomized block arrangement with three replicates. An untreated control was included as well as buffer tomato untreated rows to avoid contamination between plots. Each sample consisted of 20 randomly chosen leaflets from different plants and from different parts of the canopy before inoculation, 1 h after inoculation and every 24 h during two weeks. Fifty discs of 1.4 cm in diameter were prepared from each sample with a cork-borer. The discs were placed in 250-mL Erlenmeyer flasks containing 50 mL of sterile Triton X-100 (0.05% v/v) solution supplemented with 250 mg/L chloramphenicol. The flasks were shaken for 60 min. at 110 rpm and then 200 µL and 50 µL aliquots from the suspensions of the treatments were spread on plates containing rose bengal media. The *T. koningii* typical colonies were counted 3 days later. The regression model $\log_{10}(Y) = f(T)$ in which $Y$ is inoculum density and is a linear function of time ($T$) after application of *T. koningii* was employed to analyze the relationship between Th003 inoculum density (CFU/cm$^2$) and the time. The viability of the fungi in the suspensions was examined before treatments application. This experiment was repeated once. Since the conclusions on this repeated experiment were similar, findings of one experiment are presented in the results.

**Foliar diseases control experiment**

The biocontrol activity of the formulated and unformulated *T. koningii* Th003 was evaluated against foliar diseases naturally caused by fungi. The treatments were sprayed weekly once the foliar diseases appeared in the crop. The antagonist suspensions were used at $1 \times 10^6$ conidia mL$^{-1}$. The experimental size and spatial distribution were the same as described above, but had four replicates and included an untreated control. The foliar diseases incidence was registered as proportion of affected plants and leaves and the powdery mildew disease severity was measured in all affected leaves as percentage of leaf covered by *O. lycopersicum*, while early blight severity was measured for the lesions number on the leaves. During experiments, air temperature ($T^o$) and relative humidity (RH) were registered at one minute intervals by means of a Hobo LCD Temp./RH data logger (Onset Computer Corporation) positioned 1.5 m above the soil in the center of the greenhouse.

**Data analysis**

Statistical analysis of the data was performed using GLM and PROC CORR procedures (SAS Institute Inc.). Survival of the BCA over time was estimated by mean of regression analysis and correlated with climatic conditions. Differences in biocontrol activity were determined by using ANOVA for the area under incidence progress curve (AUDPC) and for severity disease.

**Results and discussion**

**Density patterns of *T. koningii***

No Trichoderma sp. colonies were detected in the tomato phylloplane on the sampled leaflets before treatments. Density patterns of the agent in the phylloplane was significantly different between treatments during the evaluation period ($P \leq 0.05$), being the density of the fungi higher when it was applied as unformulated conidia (Fig. 1). One hour after application, the estimated densities of *T. koningii* in the phylloplane ranged from $6.4 \times 10^1$ to $1.3 \times 10^2$ CFU/cm$^2$ for the formulation and from $1.3 \times 10^3$ to $1.5 \times 10^3$ CFU/cm$^2$ for the unformulated conidia. The density of the agent decreased by 76% at the end of the evaluation in the formulation treatment, whereas in the case of unformulated conidia treatment the density of the agent decreased 11%. The germination of the conidia in the suspensions was 18% after 24 h of
incubation and 50% after 48 h for the formulated, whereas it was 95% for unformulated conidia. On the other hand the viability of the agent expressed as CFU in the same suspensions was $8 \times 10^2$ and $1.3 \times 10^3$ CFU.mL$^{-1}$ for each treatment, respectively. This could explain the difference between the treatments in relation to the inoculum density of $T. koningii$ found in the foliar surface one hour after application, indicating an effect of the formulation process or formulation storage on the viability of the biocontrol agent. Th003 inoculum density when was applied as formulated conidia, coincided with that reported by Elad & Kirshner (1993). However, the population level of Th003 on tomato leaves in this study is considered higher compared with other studies (Freeman et al., 2004).

Figure 1. Estimated density of $T. koningii$ Th003 on tomato leaves (Log-transformed means of CFU/cm$^2$ leaf ± SE), daily temperature values, and daily humid periods at various times after application of the agent (1×10$^6$ conidia.mL$^{-1}$). The regression equations describe observations of CFU density were $Y = 1.3213 - 0.0745T$ ($R^2 = 0.40$) and $Y = 3.8852 - 0.0923T$ ($R^2 = 0.53$) for formulation and unformulated conidia treatments, respectively.

Time (days after inoculation)

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Formulation

Conidia

Max. Min. Mean

Temperature (ºC)

45 40 35 30 25 20 15 10 5 0

Log$_{10}$ CFU/cm$^2$ leaf

4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

Figure 1. Estimated density of $T. koningii$ Th003 on tomato leaves (Log-transformed means of CFU/cm$^2$ leaf ± SE), daily temperature values, and daily humid periods at various times after application of the agent (1×10$^6$ conidia.mL$^{-1}$). The regression equations describe observations of CFU density were $Y = 1.3213 - 0.0745T$ ($R^2 = 0.40$) and $Y = 3.8852 - 0.0923T$ ($R^2 = 0.53$) for formulation and unformulated conidia treatments, respectively.
Figure 2. Effect of *T. koningii* Th003 foliar application on tomato powdery mildew.
A. Incidence progress (infected leaves per plant). B. Leaf area with powdery mildew colonies. Disease was evaluated on all leaves and a mean was calculated for three canopy levels. * = significantly different from lower canopy level (older leaves) at *P* ≤ 0.05. No statistical differences were observed between treatments (*P* > 0.05).

The *T*º and RH in the greenhouse environment presented drastic variations which are very common in the greenhouses with polyethylene covering due to the deficient climatic regulation system (Fig. 1). However, inoculum density of Th003 in the tomato phylloplane presented not correlation with *T*º and RH (*r* ≤ 0.5 no significant) suggesting that others factors would have contributed to the decline of microorganism population during the evaluation period. Such factors include a reduced ability of the conidia to germinate, death of germinated conidia and physic removal of propagules (Elad & Kirshner, 1993; Yu & Sutton, 1999).

**Control of foliar diseases by *T. koningii***
The foliar diseases powdery mildew, gray mold, early blight and leaf mold caused by *O. lycopersicum, Botrytis cinerea, Alternaria solani* and *Cladosporium fulvum*, respectively, were present during the growing season. However, powdery mildew was prevalent and reached high incidence and severity levels in short time (Fig. 3), possibly due to the high
inoculum level of *O. lycopersicum* present in the untreated control as well as in the buffer untreated rows plants which also contributed to the fast development of the epidemic. Although no significant differences between treatments for AUDPC and disease severity were observed compared with the untreated control, at the end of the experiment the formulation reduced powdery mildew incidence in the tomato leaves by 25%, whereas the unformulated conidia reduced the incidence by 28%. The disease severity was reduced by formulation in 45, 66 and 60% for low, middle and upper canopy levels, respectively, 117 days after transplant, whereas unformulated conidia reduced it by 28, 40 and 59%, respectively, compared with the untreated control (Fig. 2). *B. cinerea* affected only one leaf per plant in average and the formulation reduced disease incidence by 41% whereas unformulated conidia reduced it in 45%. The early blight incidence expressed as proportion of leaves with lesions was reduced by the treatments in 36 and 37%, respectively, compared with the untreated control. We are currently studying the effect of *T. koningii* under greenhouse and field conditions in different locations.

**Acknowledgement**

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**References**


Fermentation and its influence on the survival of *Pseudomonas fluorescens* strain Pf 153 within the freeze drying process

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Abstract: For *Pseudomonas fluorescens* strain Pf 153 a freeze drying protocol without loss of viability and efficacy was developed. Afterwards, the influence of the fermentation on the viability after freeze drying was investigated. The results indicate that the fermentation time influences the viability. The application of a heat and pH shock does not result in an improvement of viability. It is also possible to enhance the biomass production by varying the liquid media. It was shown that the media did not influence the viability during freeze drying.

Key words: viability

Introduction

*Pseudomonas fluorescens* is an antagonist well known for its ability to control several plant diseases. However, limited information on the production and its influence on the formulation for the application of viable cells are available. Strain Pf 153, isolated from tobacco roots, inhibits *Phomopsis sclerotoides* when grown on PDA and KB agar. It produces extra-cellular protease and hydrogen cyanide but not 2,4-diacetylphloroglucinol or pyoluteorin (known for other *P. fluorescens* strains) as antifungal substances (Fuchs et al., 2000).

Pseudomonas are Gram negative, non-sporulating, desiccation sensitive bacteria and therefore difficult to preserve. Freeze drying is described as a successful method to preserve bacteria, yeast and sporulating fungi (Berny & Hennebert, 1991). However, bacteria survival after freeze drying varies with the species and strains (Saarela et al., 2005). In addition, external factors influence bacterial freeze drying survival: growth conditions (e.g. fermentation time, suspension medium (Saarela et al., 2005; Berny & Hennebert, 1991), cryoprotectants (Capela et al., 2006), freeze drying protocol, (Saarela et al., 2005), rehydration conditions (Carvalho et al., 2004). Therefore, it is important to investigate how different parameters influence the survival of the bacterium after freeze drying.

Material and methods

**Pre-culture**

The pre-culture was grown in KB medium for 24 h at 28°C and 150 rpm on a horizontal shaker (Novotron, Infors, CH) after inoculating with a slope of culture grown on TSA plates.

**Screening of liquid media**

In a first experiment nine standard liquid media were prepared and autoclaved at 121°C for 20 min. The autoclaved media were pipetted into a micro titre plate (99 µl) and inoculated with...
1 μl of the pre culture. Afterwards, the plates were incubated at 25°C. The biomass production was determined by measuring the optical density (OD) at $A_{595nm}$ every 24 h with an ELISA reader (Tecan-Spectra, Männedorf, CH). Based on these results three media (KB, TSB, NB) were selected and in total 25 modified media were tested like described above.

**Influence of the fermentation on the viability after freeze drying**

In four separate experiments the influence of the fermentation time, the media composition and the heat and pH shock on the viability after freeze drying was investigated. To determine the influence of the fermentation time, a liquid fermenter (ISF 100, Infors, CH) containing 3 l KB medium was inoculated with 30 ml pre culture. The fermentation was performed at 28°C, with 60 NL/h aeration and an agitation of 900 rpm. Samples were taken every four hours. To follow the bacterial growth the OD of 150 μl sample size was measured.

To investigate the influence of the media composition, three media with different carbon and nitrogen sources and ratios were selected. Flasks, containing 30 ml autoclaved media, were inoculated with 0.3 ml of the pre culture. Afterwards, the samples were shaken at 150 rpm for 16 h at 28°C. For the heat shock trials, flasks containing 30 ml of KB media were inoculated with 0.3 ml of pre culture and were then shaken at 150 rpm for 15 h at 28°C. Afterwards, the temperatures were raised to 35, 40 and 45°C for one hour. The pH shock experiments were conducted in a 3 l fermenter as described above. After 15 h fermentation, the pH was lowered of one unit every 30 min by means of HCl acid. The pH range tested was from 7 to 4.

**Freeze drying process**

Samples were suspended 1 to 1 in a non sterile 20% lactose solution. Three ml of that suspension were then freeze dried in an Advantage EL (VirTis, Gardnier, USA) freeze drier following a standard procedure. Schedule: cooling to 5°C in 20 min, freezing in two steps: to -20°C in 315 min and to -40°C in 685 min and drying at -20°C for 1080 min.

For the experiments with different liquid media and stress situations (pH, temperature) the samples were centrifuged 3 times with phosphate buffer. Afterwards, samples were adjusted to an OD of 0.95 measured at $A_{595nm}$ with a spectrophotometer (Kontron UVIKON 922) and were suspended as described above.

**Determination of the viability**

The “Most probable number” (MPN) method was used to estimate the viability of the cells before and after freeze drying. 120 μl of TSB medium were pipetted in microtitre plate cavities. After addition of 30 μl of the sample, step dilutions were performed. The microtitre plates were then incubated at 25°C for at least 48 h. The overgrown cavities were counted and the MPN was calculated (MPN Calculator Version 4.04© 1996). The freeze dried samples were re-suspended with water to the original weight before pipetted.

**Results and discussion**

When different standard media were compared, clear differences in the growth rate of *P. fluorescens* strain Pf 153 were obtained. The best growth was obtained in the KB and the TSB media. (Fig. 1). Due to the type of source, the maximum production of biomass was reached after different fermentation times. Of interest were media that permitted to reach this maximum in less time. When the best (KB and TSB) and one poor (NB) media were modified, a higher biomass production was obtained for all media.

Depending on the nitrogen source the addition of a carbon source was needed. The composition of the carbon source and the addition of different salts also influenced the bacteria growth. From the results, it was not possible to indicate one particular nitrogen or carbon source to enhance the biomass growth because combinations between the media
compounds influence differently the growth. However, it was possible to substitute an expensive nitrogen source without loss in biomass quantity.

When Pf 153 was grown in a liquid fermenter, the OD, pH and O₂ concentration where monitored over the fermentation time (Fig. 2). The OD reached its maximum after 16 h of fermentation. The oxygen concentration decreased the first 10 h, remaining constant until 17-18 h. After an increase, the oxygen content decreased slowly. The pH varied also during time. After 18 h fermentation it increased of 0.8 units. From the OD, O₂ concentration, and pH values, it was obvious that the exponential time was reached after 16 to 18 h.

The MPN value was calculated in order to measure the viability before and after freeze drying (Fig. 2). The results indicated that the viability was influenced by the fermentation time: until the end of the exponential phase a reduction of the MPN value after freeze drying was observed. The best result was obtained when Pf 153 was harvested after 20 hours.
In order to improve *P. fluorescens* Pf 153 biocontrol efficacy, the bacterium was grown in different media or a heat or a pH shock was applied. Of interest was also if these variations could influence the viability after freeze drying (Fig. 3). The statistical analysis indicated that the viability before and after freeze drying was not influenced by the liquid media and the heat shock temperature. The pH shock results were preliminary but it seemed that a pH between 7 and 5 did not influence the viability. At pH 4 reduced viability before and after freeze drying was shown.

![Graph](image1)

Figure 2. Progression curve of O2 concentration, pH and OD within one fermentation (up) and influence of the fermentation on the viability before and after freeze drying (bottom)

**Acknowledgements**

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Figure 3. Influence of different liquid media, heat and pH shock on the viability of Pf 153 before and after freeze drying. pH trials: preliminary results.
References


Necessity of highly concentrated antagonist inocula for biocontrol of Botrytis cinerea at low temperatures

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Abstract: Field trials were carried out on 7 farms in Norway to assess the ability of a commercial Trichoderma product (PlantShield) and two unf ormulated biocontrol strains, Clonostachys rosea 336 and T. atroviride P1, to control Botrytis cinerea, the causal agent of gray mold in strawberry. Prepared as recommended, PlantShield spray contained 10^6 conidia ml^-1 and the unf ormulated antagonists were applied at the same concentration. None of the treatments reduced pre- or postharvest gray mold satisfactorily. Tests on detached flowers and agar media showed that at a concentration of 10^6 conidia ml^-1, the antagonists were unable to inhibit B. cinerea at 15°C, the mean temperature during our field applications. Control of B. cinerea at 15°C necessitated antagonist concentrations of 10^7 conidia ml^-1. Awareness of the requirement for more concentrated antagonist inocula at suboptimal temperatures should improve consistency of biocontrol programs using Trichoderma and Clonostachys.

Key words: biological control, inoculum concentration

Introduction

Gray mold, caused by Botrytis cinerea, is the most important disease of strawberry (Fragaria x ananassa) in Norway. Disease in strawberry fruits often originates from infection of flower parts, which, at present, can only be prevented by repeated fungicide applications during flowering. Biological control applications also aim at preventing flower infection. There are numerous reports that biological control of gray mold can be achieved through antagonism by strains of other filamentous fungi, e.g. Trichoderma spp., and Clonostachys rosea (=Gliocladium roseum) (Tronsmo & Dennis, 1977; Elad et al., 1994; Harman et al., 1996; Köhl et al., 1998; Freeman et al., 2004; Li et al., 2004). Commercial products based on Trichoderma spp. are available to control B. cinerea in many crops, including strawberry.

To have good effect, suspensions of biocontrol agents such as Trichoderma spp. and C. rosea must be sufficiently concentrated (Hong et al., 1998; Freeman et al., 2004). Concentrations of 10^6 conidia ml^-1 of either antagonist reduced gray mold in strawberry (Peng & Sutton, 1991; Sutton et al., 1997), although 10^7 to 10^8 Trichoderma conidia ml^-1 were necessary to control B. cinerea in grapes (Dubos, 1987; Gullino, 1992; Latorre et al., 1997). When prepared according to the manufacturers’ recommendations, working concentrations of the commercial products PlantShield HC (BioWorks, N.Y.), Binab TF WP (BINAB Bio-Innovation AB, Sweden), and Trichodex (Makhteshim Ltd, Israel) are 10^6, 10^4, and 10^6 Trichoderma conidia ml^-1, respectively.

There is relatively little information on the use of Trichoderma and Clonostachys in cool climates. Both T. harzianum and C. rosea have controlled B. cinerea at 10°C in some, but not
all, trials (Sutton & Peng, 1993; O’Neill et al., 1996; Köhl et al., 1999). According to information on the product labels and the manufacturers’ web sites, the Trichoderma products mentioned above are active at temperatures over 10°C.

On the basis of the above information, we designed field trials to assess the effect of PlantShield, C. rosea, and T. atroviride P1 on incidence of gray mold in field-grown strawberries in southern Norway. Bioassays and tests under controlled conditions were also carried out to investigate the effects of temperature and antagonist inoculum concentration on biocontrol of B. cinerea.

Material and methods

Fungal isolates
The biocontrol strain C. rosea 336 was provided by Prof. J. Sutton, Univ. of Guelph, Ontario, Canada; T. atroviride P1 (Tronsmo, 1991) is a biocontrol strain from our collection, and PlantShield was provided by BioWorks, NY. Conidial suspensions of the antagonists were prepared the day of use according to manufacturer’s directions or, in the case of unformulated strains, were washed from 2-week old PDA plates and diluted to the desired concentrations.

Field trials
2-year-old fields of cv. Korona strawberries were designed as randomized complete blocks on 7 farms in southern Norway (58-62°N). Conidial suspensions of the antagonists were applied twice weekly as foliar sprays throughout the flowering period using pressure-driven backpack sprayers.

Bioassays
Detached strawberry flowers were inoculated with 10 µl drops containing conidia of B. cinerea, B. cinerea + T. atroviride, or B. cinerea + C. rosea in the concentrations indicated. Inoculated flowers were incubated in plastic boxes in high humidity and inspected daily for necrotic spots on the lower surface of the sepals. There were 3 replicates of 12 flowers per treatment, and the experiment was repeated twice.

Agar plate tests
Plates of PDFA (potato dextrose agar supplemented with 2% fructose) were inoculated with 10 µl drops of B. cinerea, B. cinerea + T. atroviride, or B. cinerea + C. rosea in the concentrations indicated. Radial growth of B. cinerea on all plates was measured daily until the control colonies reached the edge of the plate. There were 3 replicate plates of each treatment and the experiment was repeated twice.

Statistical analysis
Incidence of gray mold in the field trials was analyzed by ANOVA. Numbers of infected flowers in the bioassays were analyzed by binary logistic regression. The agar plate tests were analyzed by regression of B. cinerea colony size on antagonist inoculum concentration and incubation temperature.

Results and discussion

Field trials
Disease pressure (gray mold incidence in untreated plants) varied widely in the different trials, from 9 to 90% (Fig. 1). None of the treatments consistently reduced the amount of gray mold in the harvest, although plots treated with C. rosea generally had less gray mold than the untreated plots. The mean air temperature on treatment days at all locations was 15°C.
Bioassays

Bioassays on detached strawberry flowers indicated that both temperature and antagonist inoculum concentration affected outcome of biocontrol of *B. cinerea*. Analysis of infection data by binary logistic regression showed that at 15°C, flowers inoculated with a mixture of $10^6$ *B. cinerea* conidia ml$^{-1}$ and $10^6$ *T. atroviride* conidia ml$^{-1}$ had a probability of infection only slightly less than flowers inoculated with the pathogen alone (Fig. 2). *C. rosea* was more effective at this concentration, and when its concentration was increased to $10^7$ ml$^{-1}$, the expected probability of infection at 15°C dropped from 88% in the control to 16%.

Figure 1. Gray mold (% by weight) in harvested berries following application during flowering of PlantShield, *Trichoderma atroviride* P1, *Clonostachys rosea* 336, or a mixture of the latter two, all at a final concentration of $10^6$ conidia ml$^{-1}$. Each cluster of points represents results of field trials on different farms in southern Norway.

Figure 2. Detached strawberry flowers were inoculated with 10 µl drops of $10^6$ ml$^{-1}$ *Botrytis cinerea* conidia alone (control) or mixed with $10^6$ or $10^7$ ml$^{-1}$ conidia of *Trichoderma atroviride* or *Clonostachys rosea*. Analysis of the numbers of infected sepals in each treatment gave the expected probability of infection when inoculated flowers were incubated at different temperatures.
The ability of *T. atroviride* to prevent infection at all temperatures was most dependent on its concentration, while the performance of *C. rosea* was significantly affected both by temperature and inoculum concentration.

**Agar plate tests**

Conidia of *B. cinerea* (10⁶ ml⁻¹), alone or mixed with conidia of the antagonists, were inoculated in 10 µl drops on PDFA. Subsequent colony growth of *B. cinerea* was affected by both temperature and antagonist concentration (Fig. 3). At 15°C, both antagonists prevented growth of *B. cinerea* when they were sufficiently concentrated, i.e. 10⁷ ml⁻¹. At 25°C, much lower concentrations were effective.

Altogether, results of the field trials, bioassays and plate tests indicate that antagonist concentrations found effective elsewhere are insufficient to produce satisfactory disease control at low temperatures. Increasing the antagonist concentration should improve biological control of *B. cinerea* at temperatures suboptimal for antagonist activity.

![Figure 3. Conidia of Botrytis cinerea were coinoculated onto PDFA plates with different concentrations of conidia of Trichoderma atroviride or Clonostachys rosea and incubated at temperatures from 10 to 25°C. Inhibition was measured as colony growth of B. cinerea in the presence of antagonists relative to its growth when alone.](image)

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**Aureobasidium pullulans** (1113-5) microbial antagonist for the control of post-harvest decay on apple fruit: development of active biomass formulation at a lab scale

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**Abstract:** One strain of *A. pullulans* (de Bary) Arnaud var. *pullulans* 1113-5, was previously selected for its high antagonistic activity against *Penicillium expansum* and *Botrytis cinerea*, two molds responsible of post-harvest decay of apple fruit. The objective of the study to optimize the biomass production of this strain in a lab-scale fermentor. A dried formulation of *A. pullulans* was carried out using a fluidised bed dryer. Stability of the dried product was evaluated during storage and the antagonist activity against *P. expansum* was evaluated at a pilot scale on apple fruit. The high cell density fermentation can be achieved with *A. pullulans* using glucose fed-batch technology resulting in a final biomass dry weight of 107 g/l. A viability of 60% was measured after the drying process allowing the evaluation of this strain for a long period of storage. After 7 months of storage at 4°C, 16% of initial viability corresponding to 1.5×10¹⁰ CFU/g dry matter was noticed. The best antagonistic activity against *P. expansum* was achieved with the application of a 1×10⁸ CFU/ml suspension of *A. pullulans* on wounded fruit. We can conclude that the biomass formulation did not alter the efficacy of the biological control agent.

**Key words:** biological control, biomass production, efficacy

**Introduction**

Apple is one of the most important fruits produced in Morocco. Indeed this country contributes for 30% of the apple production in Africa, which places it in second position after South Africa. Postharvest pathogens cause substantial losses (60%) in this apple production. Fungal diseases are the major factor limiting the storage life of apples. Nowadays, synthetic fungicide treatments are the main means to limit fungal post-harvest diseases but development of resistant strains of the pathogens to many fungicides, and the growing concern for human safety and environmental protection, have resulted in attempts to develop alternative methods as the biological control of postharvest diseases (Jijakli & Lepoivre, 2004).
Aureobasidium pullulans (de Bary) Arnaud var. pullulans 1113-5, was previously selected for its high antagonistic activity against Penicillium expansum and Botrytis cinerea, two wound pathogens causing economically important losses of Golden Delicious apples during storage (Achbani et al., 2005). The yeast-like fungus Aureobasidium pullulans is one of the most widespread and well-adapted saprophytes in the phyllosphere and has been frequently considered as an effective biocontrol agent against postharvest diseases (Ippolito et al., 2000; Castoria et al., 2001). In this context, our objectives consisted in the development of active biomass formulation through the optimization of the fermentation and drying steps. Stability and antagonist activity against P. expansum of the dried product were evaluated during long term storage.

Material and methods

Biomass production
The biomass production of strain 1113-5 was carried out at 28°C in a 10 litres Biostat ® ED bioreactor (B. Braun Biotech, Germany) using fed-batch technology. Fed batch solution consisted in 50% w/w glucose solution. The medium contained per liter: 5 ml of mineral salts concentrated solution (0.32 g l⁻¹ MnCl₂ 4 H₂O, 0.49 g l⁻¹ CuSO₄ 5H₂O, 5.75 g l⁻¹ ZnSO₄ 7H₂O, 0.48 g l⁻¹ CoCl₂ 6H₂O, 0.49 g l⁻¹ Na₂MoO₄ 2H₂O, 15 g l⁻¹ EDTA, 2.94 g l⁻¹ CaCl₂ 2H₂O and 2.78 g l⁻¹ FeSO₄ 7H₂O), 30 g yeast extract, 30 g soy peptone, 0.37 g Na₂SO₄, 4.5 g K₂SO₄, 6 g KH₂PO₄, 3 g MgSO₄ 7H₂O and 700 ml distilled water. 110 g glucose dissolved in 250 ml distilled water and 5 ml vitamins concentrated solution previously sterilised by microfiltration (1 g l⁻¹ thiamine HCl, 1 g l⁻¹ pyridoxine HCl, 1 g l⁻¹ nicotinic acid, 1 g l⁻¹ D-biotin, 1 g l⁻¹ Ca-D-pantothenat, 0.2 g l⁻¹ p-aminobenzoic acid, 5 g l⁻¹ inositol) were added before inoculation. The medium was continuously aerated with 1.5 vvm, the stirring speed was maintained at 600 rpm. The pH of the culture was controlled at 5. The bioreactor was inoculated with 500 ml shake-flasks culture.

Formulation
The biomass produced in the fed batch process has been dried in a fluid bed dryer. The maize starch was used as loading agent (30% w/w). The drying process was accomplished by controlling the air temperature in the bed at 30°C and the air inflow at 150 m³ h⁻¹.

Viability and antagonistic activity
After drying, the samples were stored at 4°C, and after different periods of time (0 to 7 months), the number of viable cells was determined by plating on YEPD medium. After 7 months of storage, the antagonistic activity was conducted on Golden delicious apples by submerging in A. pullulans suspension at a concentration of 10⁶, 10⁷ or 10⁸ CFU/ml on wounded fruit (at four equidistant points). Each wound was characterised by a diameter of 2 mm and a depth of 4 mm and a distance of 20 mm inter-wounds). One day after treatment with the antagonist, fruits were pulverised with a P. expansum suspension at 1x 10⁵ CFU/ml. Each treatment was applied to three replicates of 20 fruits (240 wounds for each treatment). Fruits were placed at 5 or 25°C. The protective level and severity of decay were determined after 5 and 7 days of storage at 25°C and after 20 and 28 days of storage at 5°C. The protective levels (Y %) were calculated with respect to the following formula: \( Y \% = \frac{D_T - D_X}{D_T} \times 100 \) With \( D_T = \) diameter lesion of control and \( D_X = \) diameter lesion of treatment. All statistical analysis was performed using (SAS Institute, Cary, NC).
Results and discussion

Biomass production
The high cell density fermentation can be achieved with *A. pullulans* using glucose fed-batch technology resulting in a final biomass dry weight of 107 g/l after 48 hours of fermentation and with a yield coefficient of 0.67. The pH of the culture was controlled at 5. In those fermentation conditions, the yeast-like cell form is predominant.

Formulation
A viability of 60% was measured, after the drying process, corresponding to residual moisture of 10.5% w/w.

Viability and antagonistic activity
After 7 months of storage at 4°C, 16% of initial viability corresponding to $1.5 \times 10^{10}$ CFU/g dry matter was noticed. This viability reduction occurs in the first 30 days of storage at 4°C. After this period, the viability remains constant. The antagonistic activity against *P. expansum* showed that a protection level of 89% was achieved with the highest biomass preparation after 28 days for apples stored at 5°C and after 7 days for apples stored at 25°C (Fig. 1).

![Figure 1](image)

Figure 1. Biocontrol activity of the fluid bed-dried *A. pullulans* cells 1113-5 against *P. expansum* (880) at a pilot scale on wounded ‘Golden delicious’ apples. *A. pullulans* suspension applied at a concentration of $1 \times 10^6$, $1 \times 10^7$ and $1 \times 10^8$ CFU/ml with untreated control and *P. expansum* ($1 \times 10^5$ CFU/ml) applied after 24h. The protective level and severity of decay were based on three replicates of 20 fruit each and were determined after 5 and 7 days of storage at 25°C (left) and after 20 and 28 days of storage at 5°C (right). Columns with the same letter within the same time interval are not significantly different according to Duncan’s Multiple-Range Test, $P<0.05$ level.

In this study, we can conclude that the biomass formulation of the yeast-like fungus, *A. pullulans* (de Bary) Arnaud strain 1113-5, isolated from the surface of apple fruit, did not affect the efficacy of the biological control agent of apple post-harvest diseases (*Penicillium expansum*) after 7 months of storage at 4°C. The level of efficacy of 1113-5 at $1 \times 10^8$ CFU/ml on post harvest apple diseases obtained in our laboratory was high and opens a good
opportunity to use this strain as biocontrol agent for apple preservation. Further studies at large scale are needed to confirm these results.

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References


A microbe friendly technology to enhance survival of the biocontrol bacterium *Pseudomonas fluorescens*

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**Abstract:** *Pseudomonas fluorescens* (A506), used for control of the causal agent of fireblight *Erwinia amylovora*, was formulated using patented biopolymer technology (Patent No. 506484) to form gels, granules and water dispersible prills containing live bacteria. The survival of bacteria in formulations was monitored at 4 and 20°C. There was no loss of bacterial cell viability in the gels stored at 4°C for up to 4 months. Similarly, bacteria remained viable in granules and water dispersible prills stored at 4 and 20°C for up to 2 months. *Pseudomonas fluorescens* (F113) has biocontrol activity against *Pythium ultimum* and has been shown to reduce the severity of damping-off of sugarbeet in soil microcosms. This strain was formulated as a gel and as a coating on onion seeds. The gel formulation could be stored successfully for about 3 months with little loss in cell viability at either 4 or 20°C. The viable cell numbers on coated onion seeds remained stable at 4°C for approximately 3 months, but there was a loss in the viable bacteria numbers on coated seeds stored at 20°C. A bacterial loading of $2.1 \times 10^6$ cells per seed could be achieved on onion seeds, providing a stable inoculum for establishment of the bacteria in the soil after sowing.

**Key words:** bacterial survival, formulation, shelf life

**Introduction**

The potential for beneficial microorganisms to be used as seed coatings for the control of soil-borne pathogens, or to stimulate plant growth, has been known for several years. But, unlike chemical seed treatments, there are very few commercial products available on the market. Biological seed treatment differs fundamentally from chemical seed treatment in that the microorganisms are alive and must grow well if they are to be successful (Taylor & Harman, 1990). Biopesticides are considered to be environmentally compatible alternatives to chemical pesticides. A key to commercialization is the availability of effective formulations and delivery systems that ensure the long-term viability of the inoculant and its biocontrol activity (McIntyre & Press, 1991). There are different forms of seed treatment available, for example, dry powder, pelleting, seed priming, film coating. Most of these procedures are proprietary to the manufacturers and no information is available in the literature either on the production or stability of the products. For a product to be successful it should have high viability and vigour when delivered to the soil rhizosphere or when applied as an aerial spray to plants.

Strains of the bacterium *Pseudomonas fluorescens* have potential to be developed as biocontrol agents for control of plant diseases in crops as diverse as pip fruit, sugar beet and other crops. *P. fluorescens* has a proven efficacy in prevention of disease but variation in effect has been attributed to cell mortality during storage of this non-spore forming bacterium.
This paper describes the use of patented biopolymer technology to improve the shelf life of microorganisms during storage and enhance the delivery of robust cells to the site of action.

**Material and methods**

Strains of *P. fluorescens* were grown in shake flask culture and concentrated by centrifugation. The concentrated cells were used for the formulations.

*P. fluorescens* strain A506 was formulated either as a gel, granule, or water dispersible prills using the patented technology (Patent Number 506484). The shelf life for each of the formulations was monitored at fortnightly intervals over 4 months for gels and 2 months for granules and water dispersible prills. Biopolymer gel, containing *P. fluorescens* strain F113, was coated onto onion seed, (variety Canterbury Long Keeper) and the shelf life monitored at 20°C at fortnightly intervals. The germination efficiency of coated seeds was tested under glasshouse conditions and compared with uncoated seed. All formulations were enumerated for total viable cells counts at fortnightly intervals using standard dilution plating techniques.

**Results**

*P. fluorescens* A506 formulated as gel remained stable at 4°C for 4 months (Fig. 1), while granules and water dispersible prills retained viability for 2 months at 4 and 20°C (Fig. 2). There was an initial rapid decline in the number of viable cells in the gel formulation stored at 20°C, however cell numbers remained stable for the following 6 week period.

![Figure 1. Shelf life of *P. fluorescens* A506 gel formulation at 4 and 20°C](image)

*P. fluorescens* strain F113 formulated as a gel, was stable for 84 days at 4°C while cell numbers slowly declined when stored at 20°C (Fig. 3). Seed coating produced a high loading of cells, $4.1 \times 10^3$ bacteria/seed. Bacterial density on the coated seed remained stable for >80 days when stored at 4°C. At 20°C, bacterial numbers declined but significant number of cells still remained viable on the seed at the 70-day sampling, $3 \times 10^3$/seed (Fig. 5).

Germination efficiency was similar for both coated and untreated seeds. The initial emergence from the coated seeds was slower than untreated seed but by 14 days from sowing both treatments showed similar emergence in glasshouse tests.
Figure 2. Shelf life of *P. fluorescens* A506 granule and water dispersible prill formulations at 4 and 20°C

Figure 3. Shelf life of gel and onion seed coated formulations of *P. fluorescens* F113 at 4 and 20°C

**Discussion**

The shelf life of environmentally sensitive microorganisms continues to be a challenging and success-limiting step in the development of biocontrol products (Paau, 1998). Survival of microorganisms during the coating process and during storage at ambient temperature is critical for the development of commercial microbial products. Biopolymer technology can be used to stabilise cells of sensitive microorganisms like *P. fluorescens* although the survival characteristics may vary between strains and substrates. *P. fluorescens* A506 survived very well at 20°C when coated onto, or incorporated into, dry materials but did not survive well in a gel at this temperature. In contrast *P. fluorescens* F133 survived well in a gel but was less stable at 20°C on onion seeds. The biopolymer technology provides a flexible system for the incorporation of sensitive bacteria onto a variety of substrates and can be tailored to meet application needs.
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References


The systemic resistance induced in tomato by a non-pathogenic *Pseudomonas* strain is associated with the stimulation of the lipoxygenase pathway

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Abstract: Recognition of certain non-pathogenic rhizobacteria can trigger a systemic resistance reaction that renders the host plant less susceptible to a subsequent infection by a virulent agent. Since this induced systemic resistance (ISR) is long-lasting and not conducive for development of pathogen resistance, disease control strategies based on this phenomenon are promising.

A non-pathogenic *Pseudomonas putida* strain (BTP1) isolated in the laboratory was shown to enhance the level of resistance in bean [Ongena et al. 2004. Mol. Plant-Microbe Interact. 17: 1009-1018.]. In our work, we have demonstrated that the strain is also active on tomato since two independent experiments showed a 34% disease reduction in plants pre-inoculated at the root level with BTP1, as compared with the challenged controls. On the basis of these results, our aim is to further characterize the defense mechanisms that could contribute to the enhanced level of resistance observed in tomato upon treatment with *P. putida* BTP1.

Results from comparative biotests on TLC against *Cladosporium cucumerinum* showed an enhanced fungitoxic activity of leaf extracts prepared from BTP1-inoculated plants compared with controls before and after the infection by *B. cinerea*. This is related to the accumulation of one hydrophobic antifungal compound in infected leaf tissues induced following treatment with the bacterium. The structural characterization of this molecule is under way.

On the other hand, the lipoxygenase activity in plants treated with the bacteria was measured before and after pathogen challenge. Interestingly, the analyses revealed three-fold higher LOX activity in leaves from BTP1-treated plants as compared with control plants 48 h after the infection. Measuring fatty acid hydroperoxide-claving activity in leaf extracts showed a rapid stimulation of this LHP activity in BTP1-treated plants during the first two days after pathogen challenge to reach value (1.7-fold) higher as compared with control plants. Total RNA was extracted from tomato leaves of plants exposed to methyl jasmonate vapors for 24 h for synthesis a new cDNA of TomLoxF probe using RT-PCR technique. Interestingly, treatment with strain BTP1 was also associated with changes in *tomLoxF* gene transcription in host plants after the infection by *Botrytis cinerea* as revealed by blot hybridization analysis. This last observation has to be verified in an additional independent experiment but, collectively, our results indicate that the lipoxygenase pathway, leading to antifungal phytoxylipins, could have been stimulated in tomato plants and that this stimulation may be related to the disease protective effect afforded by the *Pseudomonas* strain.