IOBC/WPRS WORKING GROUPS:
Biological control of fungal and bacterial plant pathogens
Integrated control in protected crops, temperate climate
Integrated control in protected crops, mediterranean climate

Integration 2004

S. Michele all'Adige, Italy

Proceedings of a Meeting of the WGs:
Management of plant diseases and arthropod pests
by BCAs and their integration in agricultural systems
at
S. Michele all’Adige, Trentino, Italy
9-13 June 2004

Edited by
Yigal Elad, Ilaria Pertot and Annie Enkegaard

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Preface

The meeting of the working groups ‘Biological control of Fungal and Bacterial Plant Pathogens’, ‘Integrated Control In Protected Crops, Temperate Climate’ and ‘Integrated Control in Protected Crops, Mediterranean Climate was held in S. Michele all’Adige, Trentino, Italy (9-13 June 2004), locally organized by Ilaria Pertot and Daniele Barbacovi and entitled ‘Management of plant diseases and arthropod pests by BCAs and their integration in agricultural systems'. The meeting took place in Instituto Agrario di San Michele all’ Adige and was hosted by the SafeCrop Research Centre. The aim of the meeting was to encourage the discussion between entomologists and plant pathologists. In order to fill gaps that are evident in the field of true integrated disease and pest management, significant time was devoted to discussions on integration, holistic approaches and gaps in information and knowledge.

Thus the specific topics were:

- Integrated plant disease and arthropod pest management: possibilities for integration, problems with interactions between different tools of pest and disease management, positive and negative side effects on non-target organisms.
- Multi target agents, including both microbial and those derived from natural substances, i.e. targeting several diseases or targeting disease (s) and pest (s).
- Side effects of arthropod pest management tools on disease development and control.
- Side effects of disease control on beneficials.
- Case studies of implementation of integrated disease and pest management; successes and problems encountered.
- Integrated disease management.
- Integration of microbials and management of the greenhouse for IPM of pests and diseases according to decision support systems.
- Commercial use of microbials for pests and diseases management - present situation and prospects, including new/near registration products.
- Application of natural substances/microbials against diseases.
- Role of host plant resistance in IPM of pests and diseases.
- Induced resistance towards diseases and pests.

A big number of participants (164) from 24 countries participated in the meeting. Discussion between entomologists, plant pathologists and other plant protection experts were held. A round table discussion was one of the highlights of the meeting (Moderator: Cesare Gessler). During the round table discussion and throughout the meeting a major concern was expressed regarding the difficulties in commercialization of microbial biocontrol and other alternatives and the delay in implementation of friendly means of control. Missing information about the behaviour of biocontrol agents in production scaled up stages, during storage and the distribution chain stages and the usually short shelf life of these products were described as a drawback. The severe EU regulations for registration of microbials and the fact that the regulations are somewhat not clear at present, pose major difficulties in placing biocontrol agents in the market. The companies involved are usually small or medium enterprises and
have no strong enough financial backbones to withstand the demands. The public ignorance regarding biocontrol was also highlighted as a reason for concern among the scientists. Nevertheless, in spite of the existence of gaps of knowledge on the way to implementation of alternative control measures the support for research and development in this direction is poor.

The need for more work on integration of control methods for real life management of diseases and arthropod pests was widely expressed. Questions such as the following were raised: is there a possibility of multiple effects on targets that occur simultaneously?; is there enough knowledge regarding conflicting results - control of one problem but increase of another problem; the risk of emerging problems; the possibility of minor pathogens and pests to become important; and the limited information about the effect of alternatives on non-target organisms. A call for more research on these subjects was raised.

We wish all of us fruitful, interesting and enjoyable meetings also in the future. We acknowledge the help of Cesare Gessler, Daniele Barbacovi, Manuela Malavolta, and all the students of the SafeCrop Centre, and the support of Istituto Agrario di S. Michele, SafeCrop Centre, Provincia Autonoma di Trento, ASSOMETAB, Intrachem Bio Italia, Biotecnologie B.T., Isagro Biochem, CBCE Europe, Cantine Mezzacorona, APOT and APA Sant’Orsola

Yigal Elad, Annie Enkegaard, Cristina Castañé, Convenors of IOBC/WPRS WGs

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 biocontrol and integrated management of pests and diseases.

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BIOLOGICAL CONTROL OF FUNGAL AND BACTERIAL PLANT PATHOGENS

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INTEGRATED CONTROL IN PROTECTED CROPS, TEMPERATE CLIMATE

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In association with WG:
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Meeting Organization

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International Advisory Scientific Committee:
Claude Alabouvette, Genéviève Défago, Yigal Elad, Dan Funk-Jensen, Jürgen Köhl and John M. Whipps

Local Organization
Istituto Agrario di S. Michele all’Adige and SafeCrop Centre, TN
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Thursday  10.6.2004

Opening Session
Chair persons:  Ilaria Pertot, Yigal Elad, Annie Enkegaard

Speakers:
Gianluca Salvatori, councillor for research and innovation of Autonomous Province of Trento
Alessandro Dini, general manager of Istituto Agrario di S. Michele all’Adige
Cesare Gessler, scientific director of SafeCrop Centre
Yigal Elad, convenor of IOBC/WPRS WG Biological control of fungal and bacterial plant pathogens

Oral Session 1: Combined management for control of pests and diseases
Chairpersons:  Cristina Castañé and Gabriele Berg

Oral Session 2a: Integrated control of diseases
Chairperson:   John Whipps

Oral Session 2b: Risk characterization of BCAs
Chairperson:   Luciana Corazza

Poster Session 1
Poster Session 2

Friday  11.6.2004

Round Table: What will be the future for BCAs?
Moderator: Cesare Gessler
Participants: Elzbieta-Barbara Ceglarska, Cesare Gessler, Aleid Dik, Jürg Huber, Sergio Franceschini, Massimo Benuzzi

Saturday  12.6.2004

Oral Session 3: Management of soil borne diseases
Chairpersons:  Joeke Postma

Oral Session 4a: Mode of action of biocontrol agents
Chairperson:   Enrique Monte

Oral Session 4b: Postharvest
Chairperson:   Haïssam Jijakli

Oral Session 5a: Combination of control means
Chairpersons:  Dani Shtienberg and Jürgen Köhl

Oral Session 5b: Integrated management of diseases
Chairpersons:  Aleid Dik and Elzbieta-Barbara Ceglarska

General discussion
Moderators:   Justine Head, Yigal Elad, Annie Enkegaard
# List of Participants

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Opening session
Welcome of the Councillor for research and innovation of the
Autonomous Province of Trento

Gianluca Salvatori
Assessorato alla programmazione, ricerca e innovazione, Centro Europa, Via Romagnosi, 9
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In the Lisbon Conference of 2000 the European Union has set the goal for every member
country to reach a rate of 3% of GDP invested in research by 2010. It has been a very bold
statement, somehow forced by the understanding that just a vigorous increase of the
investments in knowledge will allow the EU to remain globally competitive in face of
countries like the US, Japan and China, growing at a much faster pace. Whereas this goal has
already been surpassed by countries as Finland and Sweden, much of the other EU members
still fall behind this line and worst of all, do not appear to be ready to meet this agreement.
This is unfortunately the case of Italy, were staunch declarations of the importance of
investing in research and innovation had to face the reality of a clear decrease of investments
in knowledge, both at the public and the private level.

In the recent years the Autonomous Province of Trento has coherently and actively
decided to pursue a different way by devoting to research and innovation ever growing public
founds, notwithstanding some initial signals of decline in its financial capabilities due to a
decrease of transfers from the central government. From 1999 to today, investments in
research ha virtually doubled and account now for 1.6% of our GDP with a 1.1% of it coming
from public sources. Compared to the rough 1% of GDP invested at the national level, this
growing figures are the best proof of a serious commitment to make research and knowledge
a cornerstone of our local development policies and our best life insurance in front of the risks
of international competitions and decline of financial resources.

Increasing the overall quality of Trentino should also be a pillar of every future plan of
internationalisation, that shall always be based on the ability of our companies as well as of
the whole system to be perceived as a credible partner for high quality, innovative and
knowledge based ventures. This is a central issue of every business mission of Trentino all
over the world, and will also be of utmost importance in our future visit to China, partly
dedicated to establishing new partnerships with research and innovation institutions.
Agricultural research is of course of the greatest importance in a system like ours where agriculture plays such an important role both socially and economically, and initiatives like yours are and will always be more than welcome as it is testified in our growing commitment to the development of San Michele.

In conclusion, let me wish you all a nice and productive conference.
Combined management for control of pests and diseases
Compatibility of intervention to control grey mould, powdery mildew and whitefly on tomato, using three biological methods.

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Abstract: *Botrytis cinerea*, *Oidium neolycopersici* and *Bemisia tabaci* are among the most important bio-aggressors encountered on tomato in greenhouses. In order to limit their development, biological methods of control have been elaborated. To protect pruning wounds against *B. cinerea* at crop level, the fungus *Microdochium dimerum* has shown a high efficacy. The plant extract from the giant knotweed *Reynoutria sachalinensis* (Milsana) has shown a high efficacy to control powdery mildews. *Lecanicillium lecanii* (Mycotal) appears to be a promising fungal biocontrol agent against whiteflies. The use of these products in a context of integrated protection of tomato requires that their efficacy is not altered when applied together. The objective of the present study was then to evaluate the compatibility of *M. dimerum* and Milsana with *L. lecanii* and vice versa. Compatibility tests were conducted on potted plants in controlled conditions. Mycotal had no significant effect on the susceptibility of tomato to either *B. cinerea* or *O. neolycopersici*, and neither *M. dimerum* nor Milsana had any effect on the susceptibility of tomato to infestation by *B. tabaci*. The efficacy of Mycotal was not altered by application of *M. dimerum* or Milsana, and the efficacy of *M. dimerum* and Milsana remained unaffected by application of Mycotal. These results suggest that these biological products could be used together to intervene in tomato greenhouses.

Keywords: *Botrytis cinerea*, *Oidium neolycopersici*, *Bemisia tabaci*, *Microdochium dimerum*, *Reynoutria sachalinensis*, *Lecanicillium lecanii*

Introduction

Grey mould caused by *Botrytis cinerea* and powdery mildew caused by *Oidium neolycopersici* are among the principal diseases encountered on tomato crops grown in greenhouse conditions. Different biological control agents have been selected to protect tomato against *B. cinerea*. Among them, spores of *Microdochium dimerum* have shown a high efficiency to protect pruning wounds against *B. cinerea* at crop level when applied directly on the wounds (Decognet et al., 1999). Various biological methods, mainly based on the utilisation of antagonistic microorganisms or plant extracts, have been evaluated for the control of powdery mildews. A plant extract from the giant knotweed *Reynoutria sachalinensis* (Milsana) has shown a high efficiency against powdery mildews on various crops including tomato (Trottin-Caudal et al., 2003) and its compatibility with *M. dimerum* on tomato was already assessed (Nicot et al., 2003). The whitefly *Bemisia tabaci* creates increasing problems in Mediterranean countries in many different greenhouse crops including tomato. Various hyphomycetes have been selected for their potential as mycoinsecticides for whitefly control. Among them, *Lecanicillium lecanii* [syn = *Verticillium lecanii*] appears to be a promising fungal biocontrol agent (Fargues et al., 2003).
The use of biological control micro-organisms *M. dimerum* and *L. lecanii* and the plant extract Milsana in a context of integrated protection on tomato requires that their efficiency is not altered when applied together. The purpose of the present study was then to evaluate the compatibility of *M. dimerum* and Milsana with *L. lecanii* and vice versa. To this end, the effect of the application of *L. lecanii* was evaluated 1) on the susceptibility of tomato to *B. cinerea* and *O. neolycopersici* and 2) on the efficacy of *M. dimerum* and Milsana against *B. cinerea* and *O. neolycopersici*. Reciprocally, the effect of the application of *M. dimerum* and Milsana was estimated 1) on the susceptibility of tomato to infestation by *B. tabaci* and 2) on the efficacy of *L. lecanii* against *B. tabaci*.

**Materials and methods**

Compatibility of the three biological methods was evaluated on potted plants of tomato cv. Raissa produced in a heated greenhouse and used when they had 8 fully expanded leaves. The monoconidial strains Bc1 of *B. cinerea* and Et1 of *O. neolycopersici* (both isolated from tomato greenhouses) were used throughout this study. Spores of *B. cinerea* were produced on PDA and aliquots of $10^4$ spores were inoculated on pruning wounds. Inoculum of *O. neolycopersici* was produced on disinfested cotyledons of *Lagenaria leucantha* cv. Minibottle maintained on Mannitol-Sucrose-Agar, and conidial suspensions were adjusted at $10^4$ spores/ml. Production of adult whiteflies was completed in closed cages containing potted tomato plants and maintained in a growth chamber at 25°C. When sufficient adults were produced, they were gently vacuumed, placed in haemolysis tubes and briefly anaesthetized with CO$_2$ for transfer. Inoculum of *M. dimerum* (isolate L13 obtained from a healthy pruning wound in a tomato greenhouse) was produced on agar medium and adjusted at $10^7$ conidia/ml. The commercial mycoinsecticide Mycotal, based on *L. lecanii*, was provided by Koppert B.V. (The Netherlands) as a formulated wettable powder. It was used as recommended by the manufacturer at the dose of 0.1% (W/V) corresponding to a spore concentration of approximately $5.6 \times 10^6$ spores/ml. The formulated plant extract ‘Milsana VP 2002’ was kindly provided by Dr. Schaette AG (Germany) and sprayed on the plants at the dose of 0.3% (V/V) as recommended by the manufacturer for greenhouse tomato production.

The effect of Mycotal on the susceptibility of tomato to *B. cinerea* and *O. neolycopersici* and on the efficacy of *M. dimerum* and Milsana was evaluated by spraying the mycoinsecticide (spore suspension) on 3 leaves per plant, 7 or 2 days before or concomitantly with pathogen inoculation. Inoculation with spores of *B. cinerea* was done on 3 petiole stubs after removal of the leaves. *M. dimerum* was applied at the dose of $10^5$ spores/wound concomitantly with *B. cinerea* on pruning wounds. Powdery mildew was sprayed as a spore suspension on the whole plant. Milsana was applied 48 hours before inoculation of powdery mildew. Reciprocally, the effect of Milsana and *M. dimerum* on the installation and development of *B. tabaci* on tomato and on the efficacy of Mycotal was estimated. Anaesthetized insects were placed in clip-cages positioned on two leaves per plant. Milsana and *M. dimerum* were sprayed 2 days before infestation to test their effect on adults’ installation on leaves. In another set of plants, the biological products were applied alone or concomitantly with Mycotal, 14 days after infestation at the second-instar larvae of *B. tabaci*. Five plants per replicate were evaluated for each treatment under study and the whole set of experiments was repeated three times. Plants were randomly distributed in controlled growth chambers with climatic conditions conducive to the development of the three bio-aggressors. For powdery mildew, disease severity (% mildewed leaf area) was estimated 21 days after inoculation. On plants inoculated with *B. cinerea*, lesion expansion on the stems (in mm) was recorded daily from the 3rd to the 7th day after inoculation. AUDPCs were calculated to compare the different
treatments and to compute a protection index. *B. tabaci* larval mortality was assessed 19 days after treatments. Larvae were examined under a binocular microscope and considered dead if they turned opaque or if mycelial outgrowth appeared. The developmental stage of each dead larva was recorded.

**Results and discussion**

**Effect of Mycotal on the efficacy of M. dimerum to control B. cinerea**

In absence of *M. dimerum*, infection of pruning wounds with *B. cinerea* was observed in all experiments. The inoculation of Mycotal had no effect on the development of *B. cinerea* (data not shown). The treatment of wounds with *M. dimerum* significantly reduced disease severity (*P*≤0.01). Mycotal had no effect on the efficacy of *M. dimerum* even when the two biocontrol agents were inoculated concomitantly on the pruning wounds (Table 1).

Table 1. Effect of Mycotal on the antagonistic efficacy of *M. dimerum* to control *B. cinerea* on tomato plants (mean values obtained from 3 independent experiments).

<table>
<thead>
<tr>
<th>Treatment against <em>B. cinerea</em></th>
<th>Myco-insecticide treatment</th>
<th>AUDPC</th>
<th>Protection index (%) (^z)</th>
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<tr>
<td>–</td>
<td>–</td>
<td>146.60 a(^y)</td>
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<tr>
<td><em>M. dimerum</em></td>
<td>–</td>
<td>34.22 b</td>
<td></td>
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<tr>
<td><em>M. dimerum</em></td>
<td>Mycotal D0(^w) wounds(^x)</td>
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<td></td>
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<tr>
<td><em>M. dimerum</em></td>
<td>Mycotal D-2 leaves</td>
<td>25.71 b</td>
<td></td>
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<tr>
<td><em>M. dimerum</em></td>
<td>Mycotal D-7 leaves</td>
<td>30.51 b</td>
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\(^w\) Mycotal applied concomitantly with *B. cinerea* (D0); 2 days before (D-2) or 7 days before *B. cinerea* (D-7).

\(^x\) Localization of application of Mycotal on the tomato plants. Wounds: application on the pruning wounds concomitantly with *B. cinerea* and *M. dimerum*; leaves: application on the leaves and inoculation of *B. cinerea* and *M. dimerum* on petiole stubs after removal of these leaves.

\(^y\) Within a column, different letters indicate significant differences based on Newmann-Keuls tests (\(\alpha=0.05\)).

\(^z\) Protection index = 100 x (AUDPC\(_c\) - AUDPC\(_t\)/AUDPC\(_c\)) where AUDPC\(_c\) represents the plants inoculated with *B. cinerea* alone and AUDPC\(_t\) the plants inoculated together with *M. dimerum* or/and Mycotal.

**Effect of Mycotal on the efficacy of Milsana to control O. neolycopersici**

In absence of Mycotal and Milsana, powdery mildew developed in the three series of experiments and disease severity varied from 3.6% to 27.8%. Mycotal had no effect on disease development (data not shown). Whatever the disease pressure, Milsana reduced drastically (*p*<0.01) the growth of *O. neolycopersici* and its efficacy was not altered by the application of Mycotal (Table 2).

**Effect of M. dimerum and Milsana on the efficacy of Mycotal to control B. tabaci**

When Milsana or *M. dimerum* were sprayed 2 days before infestation with *B. tabaci* or 14 days after infestation, no significant effect on the installation and development of *B. tabaci* was observed (data not shown). Spraying Mycotal on *B. tabaci*-infested leaves significantly increased the cumulative larval mortality and application of *M. dimerum* or Milsana did not result in any significant effect on the efficacy of the myco-insecticide (Table 3).
This study demonstrated that the two biocontrol agents, *M. dimerum* (isolate L13) and *L. lecanii* (Mycotal), and the plant extract (Milsana) were compatible with each other and could be applied in conjunction or in alternation. Therefore, these bio-products have potential to be included into an integrated protection scheme of greenhouse tomatoes.

Table 2. Effect of Mycotal on the protective efficacy of Milsana to control *Oidium neolycopersici* on tomato plants

<table>
<thead>
<tr>
<th>Treatment against <em>O. neolycopersici</em></th>
<th>Myco-insecticide treatment</th>
<th>Disease severity at 21 days after inoculation&lt;sup&gt;y&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>7.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milsana</td>
<td>–</td>
<td>0.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milsana</td>
<td>Mycotal&lt;sup&gt;x&lt;/sup&gt; D0&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milsana</td>
<td>Mycotal D-2</td>
<td>0.75&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milsana</td>
<td>Mycotal D-7</td>
<td>0.75&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>x</sup> Mycotal applied concomitantly with *Oidium neolycopersici* (D0); 2 days before (D-2) or 7 days before *Oidium neolycopersici* (D-7)

<sup>y</sup> expressed in % of leaf covered with powdery mildew

<sup>z</sup> within a column, different letters indicate significant differences based on Newmann–Keuls tests (α=0.05)

Table 3. Effect of Milsana and *M. dimerum* on the pathogenic effect of Mycotal towards larvae of *B. tabaci*

<table>
<thead>
<tr>
<th>Myco-insecticide treatment</th>
<th>Diseases treatments</th>
<th>Cumulative larval mortality&lt;sup&gt;x&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaves N°4</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>24.8 (18%)</td>
</tr>
<tr>
<td>Mycotal</td>
<td>–</td>
<td>75.4 (94%)</td>
</tr>
<tr>
<td>Mycotal</td>
<td>Milsana</td>
<td>66.7 (85%)</td>
</tr>
<tr>
<td>Mycotal</td>
<td><em>M. dimerum</em></td>
<td>68.9 (87%)</td>
</tr>
</tbody>
</table>

<sup>x</sup> mean values obtained from 3 independent experiments and expressed as angular value [arcsin√(number of dead larvae/total number of whiteflies)]. Mortality rate (%) in brackets.

<sup>y</sup>within a column, different letters indicate significant differences based on Newmann-Keuls tests (α=0.05).

Acknowledgements

This work was partly supported by a grant from INRA (PIC project). We thank Koppert b.v. and Dr. Schaette AG for providing Mycotal and Milsana, respectively, for experiments.

References


Incidence of application of an elicitor of apple tree resistance against fire blight \((Erwinia amylovora)\) on an insect pest codling moth \((Cydia pomonella)\) (Lepidoptera, Tortricidae) egg laying

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Abstract: In the frame of use of systemic acquired resistance with acibenzolar-S-methyl (ASM) against the bacteria \(E. amylovora\) we looked at the consequences on the major insect pest of apple tree \(C. pomonella\). Fifteen days after 2 sprays in June the quantities of primary metabolites (soluble carbohydrates and sugar alcohols) on the leaf and fruit surface were diminished. It was particularly visible on the bourse shoots on which the majority of insect alighted. The ASM treatment reduced the number of insect alighting (50%) and the number of eggs (60%). Experiments of egg laying in laboratory on artificial substrates confirmed the relationships between leaf surface metabolites and egg laying reduction.

Key words: \(Cydia pomonella\), \(Erwinia amylovora\), systemic acquired resistance

Introduction

The use of systemic acquired resistance (SAR) as a component in an orchard ecologically based management is expected as a potential tool of control against the bacteria \(Erwinia amylovora\). The aim of our study was to examine eventual consequences of the use of acibenzolar-S-methyl (ASM) on \(Cydia pomonella\) egg laying. We already demonstrated that acceptance of plant by this insect and its egg laying is influenced by plant metabolites, which are present on the plant surface. SAR that modifies the plant metabolism (Fritig et al., 1994) and metabolite translocation (Treutter, 2000) throughout the plant could also modify metabolite composition of the plant surface and consequently the insect behaviour. In the frame of this study we looked at the plant surface metabolite composition and at the relationships with insect behaviour.

Material and methods

**Trees and ASM treatment**

Apple trees from Golden Delicious Smoothee (2832 TR cg 10 M 106 scion VF) variety were 3 years old, 190 cm height, cultivated in containers in the open (Versailles, France). ASM was sprayed on trees on the 3\(^{rd}\) and 6\(^{th}\) of June 2003 at the concentration of 100 mg per litter of the active product corresponding to 200 mg per litter of formulated product at the “J” phenological stage (period of fruit size increase).

**Insects**

Mass reared insects (INRA of Le Magneraud (France)) were released on trees between 11 to 19 days after the treatment. Forty females were released per tree in screen cages in no choice conditions (4 replicates per treatment: non-treated and ASM treated plant). Counting of alighting and eggs were carried out from 16h to 17 h solar time just after insect release in cages.
To test the role of plant surface metabolite blends on egg laying, experiments with artificial substrates were done with single females (10x3 replicates per each treatment) in no choice conditions. Females tested had been laying eggs for two days before. Artificial substrates (white square nylon of 200 cm$^2$ and 5 µm mesh size) were impregnated with the analysed metabolite reproducing the composition of the upper leaf side of young bourse shoot of treated and non-treated plants. Concentrations of the solutions in which the clothes were soaked were such as the nylon surface contained quantities similar to those leaf surfaces with a 100-time dilution. After soaking, nylon clothes were dried under the hood at ambient temperature during about 30 min. Top, bottom, and wall of individual cages were lined with impregnated nylon cloth.

**Collect of plant surface metabolites and chemical analyses**

Collects were carried out at 17h00 solar time (just after insect observations). For one spur corymb leaves, bourse shoot leaves and fruit were sampled. Each replicate gathered two samples of each organ. There were four replicates per organ.

Collect of metabolites from the leaf surfaces used the method described by Fiala et al. (1990). For apples it was done after hiding calyx and stalk zones (25 cm$^2$ each) with paraffin. The apple median part (about 300 cm$^2$) was sprayed with 10 ml of ultra-pure water on each pole (calyx and stalk) in a rotation system in funnel form. Samples were silylated with pyridine and phenyl β–D glucopyranoside and bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). We analysed 7 metabolites: sucrose D(+)-glucose), D(-) fructose, D-sorbitol, myo-inositol, L-quebrachitol and mannitol by gas chromatography coupled to the flame ionisation detector (FID), Delsi Nermag DN 200 apparatus.

**Results**

**Eggs and alighting**

The treatment of trees with ASM reduced by 60% the number of eggs per tree (Fig. 1). The distribution of eggs within the tree on corymb, bourse shoot leaves and other leaves and apple was not changed by the treatment. The mean numbers of eggs per site were on treated and non-treated trees are seen from Table 1. Egg number was mainly reduced on the upper leaf side of the bourse shoot by the ASM treatment.

<table>
<thead>
<tr>
<th></th>
<th>Non-treated trees</th>
<th>Treated trees</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower leaf side</td>
<td>Upper leaf side</td>
</tr>
<tr>
<td>Corymb</td>
<td>2.75±0.48</td>
<td>3.75±1.18</td>
</tr>
<tr>
<td>Shoot leaves</td>
<td>8.25±2.95</td>
<td>21.0±4.8</td>
</tr>
<tr>
<td>Other leaves</td>
<td>6.25±1.44</td>
<td>9.75±2.25</td>
</tr>
<tr>
<td>Apples</td>
<td>15.75±5.19</td>
<td></td>
</tr>
</tbody>
</table>

On the treated trees the number of females alighting on the trees and coming from the cage sides was reduced by 50% vs. non-treated trees. The main reduction was observed on the upper side of the bourse shoot leaves (Figure 1). Whereas within the tree the number of alighting from site to site were similar in both treatments with a maximum observed on leaves including the bourse shoot’s and apples.
Figure 1. Reduction of eggs and alighting by ASM treatment

Figure 2. Decrease of quantities of metabolites on the upper side of the bourse shoot leaves by the ASM treatment vs. non-treated.

**Metabolite composition of apple tree organ surfaces**

Chemical analyses of metabolites of plant surface washings did not show any influence of ASM treatment except on the upper leaf side of the bourse shoots. On this site (Fig. 2) when treated there were lower quantities of glucose, sucrose, mannitol and myo-inositol which are already known as egg laying stimulant for *C. pomonella* (Lombarkia & Derridj, 2002).

There is a correlation between egg laying stimulation and metabolite quantities of the leaf surfaces. The ASM treatment impoverishes the upper leaf side of the bourse shoot leaves on which the insect mainly alights (43%).
**Influence of leaf surface metabolites of the bourse shoot leaves on egg laying**

Egg laying of females was studied on artificial substrates impregnated with the analysed metabolites reproducing the composition of the upper leaf side of the bourse shoot of treated and non-treated plants in no choice condition.

Females accepted similarly for egg laying the impregnated substrates by the blend of 7 metabolites found on the treated and non-treated bourse shoot leaves. But among females which laid eggs ≤ 10 which was similar on both treatments (ASM treated =8; Non treated = 11), egg laying stimulation was different according to the substrate. Numbers of egg per female was reduced twice on the treated bourse shoot leaf composition vs. non-treated (Fig. 3).

![Figure 3. Egg-laying on artificial substrates impregnated with 7 metabolites found on ASM treated upper leaf side surface of the bourse shoots vs. that non-treated.](image)

Such results were concordant with those obtained on trees, but do not exclude other leaf surface chemical activity on the insect egg laying response.

**Discussion**

The use of ASM diminished dramatically *C. pomonella* egg numbers on trees. It could be explained by a reduction of the insect alighting on trees and by a lower egg laying stimulation by contact. Nothing was changed concerning the acceptability by contact with plant. For the first point volatile components are probably concerned, the shape and numbers of leaves and fruit not being modified in the laps of time considered. For the second point this could be explained by the decrease of quantities of metabolites known as egg laying stimulant.

This is an example of secondary effects obtained by a SAR on a non-target organism. The modifications of the plant and particularly of its surface composition are an explanation of this effect. It would mean that the relationships between plants and organisms, which depend on plant surface composition, might be affected by SAR and particularly by ASM. This induced defence of plants has probably broader consequences than it is usually thought.
References


Using honeybees to deliver a biocontrol agent for the control of strawberry Botrytis cinerea-fruit rots

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Abstract: The present study involved the biocontrol preparation TRICHODEX (Trichoderma harzianum T39) and for treating flowers of strawberry plants against the fungal pathogen Botrytis cinerea infection and development of flower and fruit grey mould. Biocontrol of strawberry fruit grey mould by spraying the biocontrol preparation is ineffective unless very frequent applications are done or high concentrations are used. Initially we evaluated the relative efficiency of four powder dispensers in loading honeybees with the T. harzianum T39 preparation. The newly developed ‘Triwaks’ dispenser was found more effective than three other dispenser types, ‘Peng’, ‘Tub’ and ‘Harwood’, with respect to effects of the dispensers on bee activity and effectiveness loading bees with high levels of Trichoderma inoculum for a long period of time after its application. Bees leaving hives equipped with the Triwaks dispenser that was better than the others, were loaded at the most with 1.45*10⁵ cfu T. harzianum T39/bee. Strawberry flowers in a commercial polyethylene greenhouse located 24 meter far from hives with Trichoderma loaded dispenser had 3.6*10³ cfu/flower. Similar level of Trichoderma was found on flowers immediately after Trichoderma spray, but the level declined with time. These levels were found necessary for the control of strawberry grey mould. Ten beehives were placed at the edge of a commercial strawberry field and the biocontrol preparation Trichodex was added to their dispensers on a daily basis from mid December until mid March. Experimental plots were located 25-50 m from the hives. During the period of grey mould development, bee delivered T. harzianum T39 was found better than season long chemical treatment (fludioxonil+cyprodinil and pyrimethanil). It can be concluded that the delivery of Trichoderma by bees to strawberry flowers is effective in strawberry fruit grey mould control and can be integrated in an IPM scheme in this crop.

Key words: Apis mellifera

Introduction

Grey mould caused by Botrytis cinerea Pers.; Fr., is a major fungal diseases of strawberry occurring worldwide (Mass, 1998). B. cinerea, attacks flowers, setting fruitlets, mature fruits and leaves (Sutton, 1990). The main sources of inoculum for the disease in strawberry are dead leaves, mummified fruits, straw mulch (where used), neighbouring crops and weeds (Sutton, 1995). Infected flower parts, shed after bloom, adhere to the fruit surface. After infecting the flowers, the pathogen may remain quiescent until the fruit ripens (Jarvis, 1980). Diseased leaves and fruits are often covered with grey tuft consisting of mycelia, conidiophores and conidia of the fungus; eventually, the fruits rot (Maas, 1998). Chemical
control is the primary means by which grey mould is controlled. However, this may occasionally be ineffective due to the occurrence of resistant fungal populations.

Trichoderma isolates are known for their ability to control plant pathogens (Elad & Freeman, 2002). T. harzianum isolates were reported to control strawberry grey mould (Tronsmo & Dennis, 1977). In a previous study, we showed that various isolates of Trichoderma that originated from a Trichoderma collection of 76 biocontrol isolates, including isolate T-39, were effective in controlling anthracnose and grey mould in strawberry, under laboratory and greenhouse conditions (Freeman et al., 2001).

Bees are used for pollination of strawberry flowers to ensure well-shaped fruit at harvest. In addition to pollen, honey bees can transfer fungal spores and bacteria among flowers of different plant species (Batra et al., 1973). The ability of bees to vector microbes may be harnessed for biological control purposes, by using honeybees and bumblebees to transfer inoculum of fungi, bacteria and viruses from the hive to flowers. In practice, a dispenser (or insert) is attached to the hive and loaded with a powder formulation of the desired agent so foragers exiting the hive to become dusted with the agent and to deliver it to the target crop. The technique has been applied to control fire blight (Erwinia amylovora) in orchards (Johnson et al., 1993) and grey mould (Botrytis cinerea) in strawberry and raspberry (Peng et al., 1992; Yu & Sutton, 1997; Maccagnani et al., 1999; Kovach et al., 2000). It was suggested that flower serves as an infection site in these system (Sutton, 1995).

During the current work we compared different types of dispensers in the ability to disseminate inoculum onto bees, tested Trichoderma delivery to strawberry flowers, evaluated the effect of flower targeted biocontrol on fruit grey mould and finally tested the implementation of honey bees Trichoderma delivery for the control of grey mould in a commercial strawberry fields. Some of the results were published earlier (Freeman et al., 2004; Bilu et al., 2004).

Materials and methods

T. harzianum T-39 (TRICHODEX, Makhteshim, Beer Sheva, Israel; Elad, 2000), was applied in mini plots experiment using, 0.4% and 0.8% concentrations, and at 2 - 7 day interval sprays. The strawberry plants (cultivar Mal`akh), susceptible to B. cinerea were spray-treated and infected by spraying conidia suspensions of B. cinerea (10^5 conidia per ml) from 2-week-old cultures. A chemical treatment, applied once a week, using pyrimethanil (Mythos, 300 g/l SL, Bayer Crop Science, Germany) for grey mould control was also included. The control agent was either sprayed on the whole plants or directly to the flowers. Experiments were carried out during autumn and winter seasons and conducted at least two times. There were 16-24 plants per treatment, each replicated 5-6 times, set up in a randomized design. Plots were separated 50-70 cm from each other. Barriers were erected during sprays in order to prevent drift from one treatment to another.

The four dispensers that we compared in this study were referred to as “Triwaks” (developed in the laboratory of Dr. Shafir), “Harwood”, “Tub”, and “Peng”. The Harwood (Antles, 1953) and the simple open Tub are one-way dispensers, and that developed by Peng (Peng et al., 1992), and the Triwaks are two-way dispensers. The Harwood type had been used for dispersing inoculum of Erwinia herbicola to control fire blight in pear and apple (Vanneste, 1996). The Tub type had been used as a pollen dispenser for pollinating almond (Dag et al., 2000). The Peng type, which is an adaptation of a pollen-dispensing device, had been used to disseminate inoculum of the fungus Gliocladium roseum to control grey mould in strawberry (Peng et al., 1992) and in raspberry (Yu & Sutton, 1997). Using the different dispensers we tested if they affected bees activity and to what extent bees flowers were loaded.
with *Trichoderma* propagules. For this purpose bees were captured at the entrance of the hives in various distances from the hives. The bees and flowers were suspended in water containing 0.01% tween 80, shaken for 30 min, the suspension was serially diluted and aliquots of the different dilutions were spread on PDA containing chloramphenicol and rose bengale. *Trichoderma* colonies were counted 4 days later. At a later stage flowers in strawberry plots that were visited by honey bees loaded with *T. harzianum* were picked in order to test the level of the fungus on them. For this purpose we used the same washing and dilution method as mentioned above.

Under field conditions the treatments started at mid December each year. Five meter plots were sprayed on a weekly basis with either pyrimethanil or fluodioxonil+cyprodinil (Switch, 25+37.5 WG, Syngenta, Switzerland). Ten beehives were placed at the edge of a commercial strawberry field and the biocontrol preparation TRICHODEX was added to their dispensers on a daily basis from mid December until mid March. Experimental plots were located 25-50 m from the hives.

Disease incidence was evaluated. Statistical analyses of the data were performed using the JMP-in software, version 3 for Windows (SAS Institute Inc.). Differences in AUDPC of severity between treatments were determined using Fisher’s protected least significant difference (LSD) test at *P*<0.05.

**Results**

Biocontrol of strawberry fruit grey mould by spraying the biocontrol preparation on whole plants is ineffective unless very frequent applications are done or by direct application of the TRICHODEX to the flowers. The control achieved by the chemical was effective as well (Figure 1). Increasing the concentration of the biocontrol agent increased its efficacy in grey mould control when applied on a weekly basis.

![Figure 1](image)

**Figure 1.** Control of strawberry fruit grey mould by whole plant application of pyrimethanil or TRICHODEX at 2-7 days interval and by TRICHODEX application directly to the flowers. Bar=LSD

Initially we evaluated the relative efficiency of four powder dispensers in loading honeybees with the *T. harzianum* T39 preparation. The newly developed ‘Triwaks’ dispenser was found more effective than three other dispenser types, ‘Peng’, ‘Tub’ and ‘Harwood’, with respect to effects of the dispensers on bee activity and effectiveness of contaminating bees with high levels of *Trichoderma* inoculum for a long period of time after its application. We found differences in these parameters of performance between dispenser types, with the Triwaks dispenser having the overall best performance. Bees leaving hives equipped with the Triwaks dispenser were loaded at the most with 1.45*10^5 cfu *T. harzianum* T39/bee. This dispenser was further tested to evaluate the population level of *T. harzianum* T39 on flowers...
visited by the bees. Strawberry flowers in a commercial polyethylene greenhouse located 24 meter far from hives with *Trichoderma* loaded dispenser had $3.6 \times 10^3$ cfu/flower. Similar level of *Trichoderma* was found on flowers immediately after *Trichoderma* spray, but the level declined with time. These levels were found necessary for the control of strawberry grey mould. Thus, bees proved to be a potential means for the delivery of the biocontrol fungus to the infection site of *B. cinerea*.

During the period of grey mould development, bee delivered *T. harzianum* T39 was found to be as good as (2003 experiment) or better (2004 experiment) than season long chemical treatment (fludioxonil+cyprodinil and pyrimethanil) (Figure 2).

Figure 2. Control of strawberry grey mould by TRICHODEX delivered to flowers by bees, by weekly applications of chemical fungicides or by both

**Discussion**

Similar to earlier report, we found that the protection of strawberry flowers is crucial in order to prevent the infection and development of strawberry fruit grey mould. Sprays under controlled conditions protected the flowers from infection and resulted in reduced fruit rot when a chemical botryticide was used and when the biocontrol agent TRICHODEX was applied at high concentration, at frequent applications or targeted directly to the flowers (Figure 1).

The ability of honey bees to vector microbial agents can be harnessed for biological control purposes, by using them to transfer inoculum of fungi and bacteria from the hive to flowers. In the present study bees were found effective for delivery of the biocontrol preparation TRICHODEX (T. *harzianum* T39) to flowers of strawberry plants against the fungal pathogen *B. cinerea* infection and development of flower and fruit grey mould. It can be concluded that the delivery of *Trichoderma* by bees to strawberry flowers is effective in strawberry fruit grey mould control and can be integrated in an IPM scheme in this crop.

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Laboratory evaluation of antifeedant activity of *Trichoderma* spp. isolates in aphid biocontrol

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Abstract: Fungal isolates of two species of the genus *Trichoderma* were evaluated as antifeedant compounds towards winged and wingless morphs of the aphid *Schizaphis graminum* and wingless morph of *Megoura viciae*. The three tested isolates showed repellence towards winged morph of *S. graminum* and wingless of *M. viciae*; only *T. citrinoviride* proved to be repellent towards wingless of *S. graminum*. Electrophysiological studies showed that the structures involved in the perception of the fungal metabolites are located on the aphid tarsomeres supporting the hypothesis that at least part of the fungal metabolites responsible for the repellent effect are water soluble compounds.

Key words: biocontrol, repellence, taste sensilla, electrophysiology, scanning electron microscopy

Introduction

Fungi offer several potential strategies against pest insects to reduce crop losses. Indeed, fungi and their metabolites can influence, by different modalities, several traits of the insect biology, such as survival, development, fecundity and feeding behaviour, but very few data are available on the repellency of fungi towards insect pests (Ekesi et al., 2001; Ganassi et al., 2004). We started a laboratory study to evaluate the potential of some fungal isolates belonging to different species of the genus *Trichoderma* (Mitosporic Fungi) as antifeedant compounds towards aphids. The final goal aimed to recognize the aphid structures involved in the perception of fungal metabolites with repellent effect.

Materials and methods

*Test organisms*

Aphids belonging to the species *Schizaphis graminum* (Rondani) and *Megoura viciae* Buckton were maintained in the laboratory on wheat plants (*S. graminum*) or on broad bean (*M. viciae*), in a thermostatic chamber at 20°C under L16:D8 photoperiod to induce parthenogenesis. The trials were carried out utilising winged and wingless adults of *S. graminum* and wingless of *M. viciae*. Winged were obtained in the laboratory by crowding.

Isolates of the genus *Trichoderma* utilised for the trials were: *T. harzianum* ITEM 908, *T. harzianum* ITEM 4485 and *T. citrinoviride* ITEM 4484. These isolates were obtained from soil or plants from Apulia and were maintained in the “Istituto di Scienze delle Produzioni Alimentari” ISPA CNR–Bari. Each isolate was cultured on 200 g of autoclaved rice kernels in 500-ml Erlenmeyer flask and inoculated with 10 ml of an aqueous suspension containing...
approximately 10^7 conidia/ml. Cultures were incubated at 25°C under a L12:D12 photoperiod for four weeks. The harvested culture material was dried in a forced draft oven at 35°C for 48 h, finely ground to 0.2 mm grain size and stored at 4°C until use.

**Antifeedant tests**
The fungal formulations of each isolate, prepared as described before, have been suspended in sterile distilled water, shaken for 20 minutes, and tested at the concentration of 50 mg/ml. A suspension of rice kernels finely ground (50 mg/ml) was also used. Excised leaves were dipped in suspensions or in distilled water for ten seconds. The leaves were placed on wet filter paper in 12 cm Petri dishes. In each dish two leaves, one treated with fungal or rice suspension (T) and one dipped in distilled water (C), were arranged in parallel with 4 cm separation. Aphids were placed with a fine brush in the centre of the dish, between the two leaves, and their position was registered 1, 3, 6 h after initial access. Walking and stationary insects on leaves were not discriminated. Per each test 10 aphids were used and 12 replicates were made (total aphids per treatment = 120). Data were subjected to Mann-Whitney U test.

**Electrophysiological experiments**
Winged and wingless morphs of *S. graminum* and wingless of *M. viciae* were used. Antennal responses were obtained using a standard electro-antennographic technique (De Cristofaro et al., 2000). Electrophysiological responses from sensilla on the mesothoracic distal tarsomere were recorded combining different equipments and techniques previously used to study single chemosensory sensilla (Hodgson et al., 1955; Den Otter, 1992; Den Otter et al., 1996; Solinas et al., 2001). The indifferent electrode, a glass micropipette with tip diameter of 5 µm, filled with a Beadle-Ephrussi saline, was inserted into aphid prothorax. The recording electrode, a glass micropipette with tip diameter of 2 µm, containing one of the test stimuli (50 mg/ml of a formulation or ground rice in 100 mM NaCl aqueous solution) or 100 mM NaCl alone (control), was contacted to the sensillum tip. Electrical connection was obtained by silver wires inserted into the glass pipettes and attached to an electrophysiological equipment (INR-01, Syntech, The Netherlands). Concentrations were chosen according to preliminary tests and behavioural responses. The test solutions were put in the micropipette 10 s before the experiment. Single sensillum recordings were carried out at 22±2°C and 70±10% R.H. Electrical activity was recorded for 1 s after stimulus onset and 5 min was allowed to elapse between presentation of successive stimuli to the same sensillum. Test and control solutions were applied in a random series on the same sensillum. Action potentials (spikes) were recorded on a magnetic tape (Sony, CditII, IEC II/Type II, High Bias 70 ms EQ, position chrome) by a double channel recorder (Sony, TC-D5M) and analysed with AutoSpike™ 3.1 (Syntech, The Netherlands). Sensilla that failed to respond to the tested solutions were considered not functioning and discarded (Crnjar & Prokopy, 1982). Responses of the sensory cells were evaluated as spike frequency (spikes/s) during the first second of stimulation, 100 ms after stimulus onset. Firing frequencies were compared by Student’s t-test.

**Scanning electron microscopy**
Excised legs of winged and wingless of both species were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4°C, dehydrated in a graded ethanol series, critical point dried in a Critical Point Dryer 010 (Balzer), gold-palladium coated to a thickness of 10 µm in a S.E.M. Coating Unit E 500 (Polaron) and observed in a Philips XL-40 at the “Centro Interdipartimentale Grandi Strumenti” of the University of Modena and Reggio Emilia.
Results and discussion

A repellent effect of three tested isolates was detected towards winged morphs of *S. graminum* and wingless of *M. viciae* (Table 1). A different sensitivity was observed across the winged and wingless morphs of *S. graminum*, indeed only *T. citrinoviride* proved to be repellent towards wingless. In terms of the sensory organs involved in the perception of metabolites, electro-antennographic studies showed that volatile compounds of isolates were not able to stimulate the olfactory sensilla of both aphid species; as a consequence these substances cannot be responsible for the repellent effect.

Table 1. Mean number (± SD) of winged and wingless morphs of *S. graminum* and wingless morph of *M. viciae* observed at different times on leaves treated with fungal formulations, or ground rice kernels (50 mg/ml) (T) or water (C)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Winged</th>
<th>Wingless</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizaphis graminum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (h)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Winged Isolates</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td><em>T. harzianum</em></td>
<td>4.2±1.3</td>
<td>0.7±0.9 **</td>
</tr>
<tr>
<td><em>T. harzianum</em></td>
<td>4.0±1.6</td>
<td>2.1±1.1 **</td>
</tr>
<tr>
<td><em>T. citrinoviride</em></td>
<td>3.8±1.7</td>
<td>1.2±1.0 **</td>
</tr>
<tr>
<td>Rice</td>
<td>3.0±0.6</td>
<td>3.5±1.4 n.s</td>
</tr>
<tr>
<td>Wingless Isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. harzianum</em></td>
<td>3.5±1.6</td>
<td>3.2±1.3 n.s</td>
</tr>
<tr>
<td><em>T. harzianum</em></td>
<td>4.3±1.3</td>
<td>3.2±0.6 n.s</td>
</tr>
<tr>
<td><em>T. citrinoviride</em></td>
<td>5.2±1.0</td>
<td>2.3±1.6 **</td>
</tr>
<tr>
<td>Rice</td>
<td>3.7±2.0</td>
<td>3.9±1.8 n.s</td>
</tr>
</tbody>
</table>

Two sensory cells of the taste sensilla lodged on the mesothoracic distal tarsomere of both aphid species were more strongly stimulated by aqueous solutions of fungal formulations than aqueous solutions containing the rice ground alone (Table 2).

The results confirm that the structures involved in the perception of the fungal metabolites with repellent activity are located on the aphid tarsomeres and also support the hypothesis that at least part of the fungal metabolites responsible for the repellent effect are water soluble compounds. SEM observations pointed out a lower number of papillary sensilla
in wingless as compared to winged morph of *S. graminum* and this could explain the different sensitivity of two morphs.

Table 2. Spike frequency (spikes/s ± SD) recorded from cells with different action potential amplitude of *S. graminum* and *M. viciae* tarsal sensilla on stimulation with fungal formulations, or ground rice kernels (50 mg/ml) in NaCl aqueous solution (100 mM)

<table>
<thead>
<tr>
<th></th>
<th><em>Schizaphis graminum</em></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Winged (n=8)</td>
<td>Wingless (n=10)</td>
<td>Wingless (n=8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
</tr>
<tr>
<td><em>T. harzianum</em> 908</td>
<td>16.6±1.8 b</td>
<td>18.6±1.2 b</td>
<td>3.6±1.6 a</td>
<td>5.6±1.6 a</td>
<td>38.4±3.2 b</td>
</tr>
<tr>
<td><em>T. harzianum</em> 4485</td>
<td>28.6±2.8 c</td>
<td>34.6±1.8 c</td>
<td>8.4±1.6 b</td>
<td>6.4±1.8 a</td>
<td>42.8±3.8 b</td>
</tr>
<tr>
<td><em>T. citrinoviride</em> 4484</td>
<td>15.8±1.8 b</td>
<td>8.8±2.4 a</td>
<td>3.4±1.8 a</td>
<td>5.8±1.4 a</td>
<td>12.6±2.8 a</td>
</tr>
<tr>
<td>Rice</td>
<td>7.6±2.2 a</td>
<td>8.6±1.8 a</td>
<td>4.0±2.0 a</td>
<td>6.0±1.4 a</td>
<td>11.8±1.6 a</td>
</tr>
<tr>
<td>NaCl</td>
<td>6.4±2.0 a</td>
<td>7.4±2.0 a</td>
<td>3.4±1.8 a</td>
<td>5.4±1.8 a</td>
<td>10.4±2.2 a</td>
</tr>
</tbody>
</table>

Different letters on the same column show significant differences (P=0.01) Student’s *t*-test

References


Biological control in chestnut cultivation:
criteria for a sustainable management

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Abstract: Chestnut stands are still an important resource for European mountain regions either for fruit and wood production or for landscape and recreational value. Diseases and pests can severely affect cultivation but their control needs to respect the ecological equilibrium of this stands which are mainly forest ecosystem. In the last years researches carried out in Italy and in other European country were focused to define criteria to obtain a good biological control of the main phytosanitary problems: chestnut blight, ink diseases and cydia damages. The natural spread of hypovirulent strains of Cryphonectria parasitica has enhanced the spontaneous recovering of many chestnut stands and now silvicultural management, artificial inoculation and biological wax for grafts protection are available to maintain or increase the hypovirulence effectiveness. Soil management by means of manuring proved to be useful in the control of ink disease foci, aiming to restore natural competitors to Phytophthora. Pheromones for cydiae monitoring were improved recently and techniques of mating disruption are going to be tested. The possibility to have cheap and effective biological control methods is needed to maintain and increase chestnut cultivation sustainability

Key words: Castanea sativa, Cryphonectria parasitica, Phytophthora cambivora, Cydiae, sustainable management

Introduction

Chestnut cultivation is coming back to be an important resource for several mountain areas in Italy and in Europe. The recovery of traditional production of chestnut and especially of “marrons” is strictly related to the increasing demand of environmental friendly products, which characterise our highly industrialized and urbanized countries (Pettenella, 2001): so these traditional foods for poor people are now regarded as highly natural products, related to precious and healthy landscape in the mind of consumers. This fundamental change in the product image has enhanced increasing price able to stimulate the recovering of cultivation in many stands: now chestnut “marrons” trees can be considered as one of the more remunerative fruit tree for mountain. Besides, chestnut stands still play a fundamental role as landscape component and as wood production, enhancing both touristic use and local furniture production. Other secondary products as mushrooms and game play a strong local economic role. This new economic perspective of these old and traditional cultivation need to be supported by an effective control of phytosanitary problems, with techniques able to save the environmental characters of chestnut orchards, really more forest ecosystem than agricultural ones. To protect trees and nuts saving both environmental and economical
sustainability is one of the goals pursued by the researches carried out by IPP Institute of CNR and by IASMA, respectively on pathogens and pest in chestnut ecosystems. In this paper we resume the result of this research regarding the three more important phytosanitary problem: chestnut blight, ink disease and cydia infestations.

**Chestnut blight**

*Cryphonectria parasitica* (Murr.) Barr can now be considered as naturalized if not endemic in almost all the natural range of *Castanea sativa* (Turchetti & Maresi, 2000). After the strong impact of the first epidemic stages, following the recovering of the disease in 1938, the appearance of abnormal cankers, due to hypovirulence and first observed in 1950 (Biraghi, 1953), changed completely the destiny of European chestnut. In fact the hypovirulence natural spread allowed the recovering of most of the affected stands and enhanced the chances of surviving of even the bad damaged trees. The recovering of cultivation was enhanced too because of the strong reduction of damages following the management practices as pruning and thinning.

Data of a field survey confirm this general good situation and the predominance of healing and healed cankers due to hypovirulence: investigation carried out in Lombardia (Davini et al., 1998) showed that abnormal and intermediate cankers were the 92% of the infection observed on 17.000 trees surveyed. Similar percentages were recorded in almost all the visited stands and orchards of different Italian and European areas. In these situation blight damages due to normal cankers were limited to dominated and stressed sprouts or trees (Turchetti & Maresi, 2000) while more vigorous plants can host several abnormal infection without reduction of growth and fruit production. In healing and healed cankers the growth of *C. parasitica* mycelia is limited to the external part of the bark and cambium is not affected at all by the infection, so it survives still producing new tissues (Figure 1). In normal cankers the fungus kills the cambium, tissues sunk and the plant dies when the infection girdles the affected branch or trunk.

The abnormal cankers are due to the presence of hypovirulent strains in the fungal population. Morphological and behavioural characteristics of hypovirulent strains are related to the presence of dsRNA hypovirus (Hillman et al., 1995). Transmission of hypovirus between fungal strains by means of hyphal anastomosis proved to be effective in the European population of *C. parasitica*, which is characterised by a reduced vegetative compatibility (v-c) groups number (Cortesi et al., 1996). Besides, European hypovirulent strains show a large compatibility range (Maresi et al., 1995). Some of v-c groups are very common in most of the chestnut range even if new ones are regularly recorded when more detailed investigations are carried out in limited areas. Till now, the appearance of new v-c groups was not related to new damages foci and the effectiveness of hypovirus transmission seems still confirmed. Pycnidia produced on healing and healed canker are able to spread both virulent and hypovirulent conidia in different rates. In this mixed inoculum, after the first stages of germination, hyphal anastomosys are produced and hypovirus can spreads throughout all the mycelium during the growth in the bark. Resistance of *Castanea sativa*, less susceptible to blight than *Castanea dentata* and probably different degrees of pathogenicity between virulent strains could be also supposed as other factors involved in the winning spread of hypovirulence (Turchetti & Maresi, 2000).

Removal and destruction of normal infection is a easy way to reduce the virulent inoculum while all the healing and healed cankers need to be released: in fact few pycnidia are produced in these infection, so only a large number of abnormal cankers seems able to assure the predominance of hypovirulent inoculum. It is possible to obtain this goal where the disease is still dangerous by means of combined artificial inoculation of selected hypovirulent strains, which are able to develop in healing cankers producing pycnidia and hypovirulent
conidia (Turchetti & Maresi, 1991). This biological treatment proved to be a cheap and easy way to obtain a good amount of hypovirulent inoculum (Antonaroli & Maresi, 1995) while curative treatment of developing infection as suggested by Grente & Berthelay-Sauret (1978) showed a quite good performance but appeared too expansive for a wide use. “Let’s hypovirulence work” is the goal of a right management of chestnut stands, based on the traditional silvicultural treatment, with the constant removal of dead branches and sprout and the release of the abnormal infections: doing so, most of Italian chestnut growers are able to check the disease with damages so few that in some areas blight is no more considered a real worry.

We can consider now blight as a component of chestnut ecosystem: the balance between host and the fungus is strongly influenced by hypovirus or hypovirulence presence. This balance can be altered by other constrains factors as drought, jail, windstorm or games and livestock damages: some new foci of virulence were related to these factor but, till now, they were never able to start a new epidemic wave and hypovirulence seemed able to check also these perturbation within few years. Of course, the appearance of new lines of the pathogen is continuous and the risk of new v-c groups unchecked by hypovirulent strains is possible. The continuous monitoring of damages is so another important practise to follow the evolution of the disease.

In the favourable condition of hypovirulence presence, grafts can be still badly damaged by blight attacks, because of the weakness of scions unable to react also to hypovirulent strains infections. After field observation and laboratory tests of antagonism, a wax containing a biological additive able to check the development of \textit{C. parasitica} was patented (CNR patent 9406) and it is now a commercial product (\textit{Cerafix plus}) (Santagata et al., 1996). A good performance was observed during grafting carried out by chestnut growers in several different chestnut orchards for many years. Of course, a correct graft execution and good scions storage are still fundamental.

\textbf{Ink disease}

This disease has been known since XVIII century in Italy but the casual agent, \textit{Phytophthora cambivora} (Buism.) Rand. was identified only in 1917 by Petri (1917). At the beginning of the XX century the disease was the main problem for chestnut cultivation: the appearance of blight and the abandon of mountains and of chestnut cultivation lose interest in this pathology.
that was not reported or studied for several years. New attacks were recorded in the eighties and nineties (Turchetti & Parrini, 1993; Anselmi et al., 1996) when the disease appeared in the recovered orchards causing the death of several trees. Symptoms are a suffering crown with smaller and yellowed leaves, burrs only on the top of the canopy, characteristic browning of the collar area and death of trees sometimes in first season of appearance of symptoms, sometimes in few years. The reappearance of the ink disease in Italy could be due to the climatic trends in the last 10-15 years: suffering of roots during repeated drought periods could have enhanced fine root infections in the following rainy months. Investigations carried out in Tuscany (Turchetti et al., 2000) have proved that the pathogens is present in several stands, not only in the bottom of valleys but also on slope and ridge, apparently without link to water distribution, as though in the past. The characteristics of this root rot disease and the mountain environment of many of the affected stands don’t allow the use of chemicals which are of difficult application and high pollution risk on water, soil and fruit.

The fungus is an inhabitant of soil and several studies suggest a strong antagonism towards mycorrhizal fungi (Branzanti et al., 1994). Starting from these considerations and trying to enhance the soil competition against *P. cambivora*, tests were carried out in field condition using manuring of trees showing the first symptoms of the disease. Chicken drops, manure and organic amendment were used together at the beginnings of vegetative season (Turchetti et al., 2003). As reported in Table 1, after three years of treatments most of the treated trees are still alive and show a good recover. Better soil management techniques are also proposed as drainage of moist soil and composting of burr and leaves with following release of this good organic matter under the crowns of trees. Further investigation on effect of manuring on soil microbial populations are going to be carried out.

Table 1. Percentages of surviving trees, suffering for ink disease attack and treated with manuring; data from Turchetti et al. (2003): 350 trees surveyed.

<table>
<thead>
<tr>
<th></th>
<th>dead %</th>
<th>suffering %</th>
<th>recovering %</th>
<th>recovering full %</th>
</tr>
</thead>
<tbody>
<tr>
<td>treated</td>
<td>7</td>
<td>2</td>
<td>20</td>
<td>71</td>
</tr>
<tr>
<td>untreated</td>
<td>13</td>
<td>87</td>
<td>–</td>
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</table>

*Cydiae*

The presence of *cydiae* is a well know problem on nuts production in chestnut orchards. Investigations carried out by IASMA (Angeli 1995; Angeli et al., 1997) proved that three different species affected chestnut. While *Pammene fasciana* appears at the beginning of summer and act as a natural regulator of burrs density, both *Cydia fagiglandana* and *Cydia splendana* attack maturing burrs and can damage from 10 to 35-40% of production depending from season behaviour. This percentage can be very effective especially for small farms. *C. fagiglandana* appears at the end of July while *C. splendana* flies between August and September (Figure 2). Damage is caused by the fall of burr, by the penetration in the fruit and by external rosure of nuts. The damages are variable in the different years and in the different locality: it is to notice that these lepidopterists are a normal component of chestnut and mixed woods and so it is impossible to obtain their complete elimination from the orchards which are generally located in these woods.
Chemicals treatments are not proposable either for the environment impact or the costs and practical difficulties because of big trees and not mechanizable stands. Till now, good results were obtained from the monitoring: flight period as like as the best pheromones mix were set up by research (Angeli et al., 1997, 1998). On this basis, new perspective were now investigated: IASMA is now studying the use of sexual confusion and disorientation as like as attract and kill strategy following the good result obtained in apple orchards and in vineyard where these biological control strategies performed very well in the last years. Many practical problem arise considering the structural characteristic of old chestnut stands, with high trees difficult to climb, and also the bio ecology of Cydiae: in fact most of pest captures happened in the upper part of crown between 4 and 8 meters. The availability of right and effective pheromones mix, now commercialised by different European company, is encouraging the study and the research on this subject. The hope is to obtain in the next years a simple biological control methods able to check the presence of Cydiae to a small level of damage. Traditional practises to manage burr and nuts in post harvest are still an useful way to control this problem.

**Conclusion**

After several years of work, based mainly on field observation, simple, effective and cheap control techniques area available to support a sustainable management of chestnut orchards. The comprehension of hypovirulence as an ecological phenomenon based on a bio molecular mechanism has opened new perspectives allowing the recovering of cultivation and its maintenance without any chemical treatment. This effective biological control mechanism appears unique and, till now, no exportable in other pathosystem, even if several studies are carried out on hypoviruses. Ink disease could be checked by the right soil management practices and by the use of organic manuring to enhance the action of soil antagonists. On this subject a better comprehension of fungal biology is one of the goal of research. Control of cydia damages is a need becoming more and more important. The availability of effective pheromones mix suggest that good results could be reached in few years even if it is to test a right techniques able to overcome all the difficulties due to the chestnut orchards.
characteristics. Education of technicians and growers is a fundamental step to enhance the introduction of adequate techniques. Know and understand the chestnut stands as an ecosystem is the way to obtain a good control of phytosanitary problem and a real sustainable management. It is to notice that only a continuous monitoring of stands by growers and techniques will permit to check any change in phytosanitary conditions.

References


Integrated control strategies for all pests and diseases in several glasshouse crops and implementation in practice

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Abstract: In The Netherlands, an inventory on all measures against pests and diseases in glasshouse crops has been made. The compatibility of various cropping practices and pesticides is being studied. In large-scale glasshouse experiments, different combinations of control strategies against the most important pests and diseases have been tested in cucumber, chrysanthemum and rose. In cucumber, powdery mildew was the most important yield-reducing factor. It can be controlled by using partially resistant cultivars. A new natural product was also effective against powdery mildew. In chrysanthemum, aphids were the biggest problem and completely non-chemical control is not yet possible in this crop. In rose, powdery mildew control was feasible with natural products. Natural pest control was also effective. The best control strategies are currently tested by groups of commercial growers.

Keywords: biocontrol, chrysanthemum, cucumber, pests, powdery mildew, rose, tomato

Introduction

Traditionally, plant protection research has focussed on preventing and controlling one single disease or pest at a time. This has lead to integrated and biological control strategies against a single pathogen or pest animal, which in glasshouses may include the use of natural enemies and/or biological products based on micro-organisms or pesticides of natural origin (PNO’s) as well as the choice of cultivar, climate control and cropping practices. In many cases, a combination of all these factors will improve control.

From a systems-approach, knowledge on interactions between the different control measures in a system is needed in order to implement successful control strategies for more than one (preferably all) pest(s) and disease(s). Therefore, an inventory was made of all effective measures against all pests and diseases (+ the effect on non-targets) for the ten economically most important glasshouse crops. Secondly, compatibility between control compounds and natural enemies was established in lab-scale trials. Thirdly, different strategies against several pests and diseases are compared in large-scale glasshouse trials. Fourthly, an implementation project has started in which all state-of-the-art knowledge of growers, researchers and advisors is combined. In this paper, trials in cucumber (autumn 2002 and spring 2003), chrysanthemum (summer 2003) and rose (autumn 2003) in glasshouses of Applied Plant Research in The Netherlands will be discussed. Several strategies, which included various chemical and non-chemical measures, were compared for their efficacy against all pests and diseases and on the quantity and quality of the harvested products.
Material and methods

Experimental design

**Cucumber**

Experiment 1: four glasshouses were planted with cucumber. One was set at standard climate, one received artificial light (10,000 lux artificial light for 12-20 h per day), one was set at humid conditions (ventilation T 4°C above heating T) and in one greenhouse a powdery mildew (PM)-resistant cultivar was used. In each greenhouse, 4 blocks of 6 treatments were laid out: 1. untreated; 2. integrated control of powdery mildew; 3. integrated control of Botrytis; 4. integrated pest control; 5. integrated control of pests and diseases; 6. standard chemical control.

Experiment 2: three glasshouses were each planted half with cultivar Euphoria and half with cultivar Grendel (partially resistant to PM). The standard conditions, artificial light and humid conditions were set as in experiment 1. In each cultivar in each glasshouse, one half was treated with ‘standard chemical control’, one half with ‘maximum biological control’. In the biological strategy chemical pesticides were only used when the biological products were not sufficiently effective.

In cucumber, Sporodex (*Pseudozyma flocculosa*, Plant Products LTD, Canada), KBV99-01 (an experimental product based on a natural antimicrobial system, Koppert B.V., The Netherlands, only used in experiment 2) and NaHCO₃ were used against PM, Trichodex (*Trichoderma harzianum*, Mahkteshim Agan LTD, Israel) was used against Botrytis. Mycotol+Addit (*Verticillium lecanii*+an additive based on vegetable oil, Koppert BV, The Netherlands) and Inseclear (fatty acids from plant oil, Ecoprotecta BV, The Netherlands) were used against pests. Chemical control consisted of regularly used pesticides. In both experiments and all glasshouses natural enemies were used against thrips and if necessary against white flies and aphids.

**Chrysanthemum**

In a glasshouse of 730 m², four control strategies were compared in cultivar Euro: 1. chemical (risk-avoiding, early applications and including the preventive use of Aaterra (a.i. etridiazol) against Pythium); 2. biological with natural enemies and PNO’s (only PNO’s which can be used by organic growers); 3. integrated with natural enemies and chemical correction; 4. integrated as 3. but including leaf application of nematodes (Nemasys F, a.i. *Steinernema feltiae*, Becker Underwood, Great Britain).

**Rose**

In a glasshouse of 307 m², five control strategies were compared: 1. untreated; 2. predatory mites, gallmidges and parasitoids or natural enemies against pests, Sporodex and KBV99-01 against PM; 3. chemical; 4. biological with natural enemies and Sporodex; 5. integrated: natural enemies, KBV99-01 and chemical correction.

**Assessments**

All pests and diseases were assessed with 7-14 day intervals. Measures were taken based on the assessments. Scouting of pests was done on both plants and yellow sticky cards. Yield was recorded, both quality and quantity.

**Statistical analysis**

Disease severity, pests and yield variables were analyzed by analysis of variance, followed by Fisher’s protected LSD test at P<0.05. Regression analysis was done on the Area Under the Curve (AUDPC) for yield against PM severity in cucumber.

**Environmental impact**

Environmental impact was calculated with the system of the Dutch organization CLM (http://library.wur.nl/milieumeetlat/glas.html).
Results

Cucumber: in both experiments, PM was the most important problem. Artificial light increased PM severity and even chemical control was not sufficiently effective. Side effects were found of control measures on non-target pests, for example Mycotal + Additon on PM. Yield was mostly affected by PM severity (Figure 1). Humid conditions decreased plant death due to Botrytis in the first experiment.

In Experiment 2, PM occurred first in the glasshouse with light in the susceptible cultivar. The biocontrol agent and NaHCO$_3$ were less effective in this cultivar than in previous experiments (Dik & Wubben, 2002) due to high disease pressure. The product KBV99-01 seemed effective but was only used when severity was already high. No differences occurred in efficacy of pest control between strategies. Yield was affected by PM in the susceptible cultivar and was higher in the chemical strategy (Table 1).

Figure 1. The effect of PM severity on yield in cucumber glasshouses set at different conditions

Table 1. Comparison of input of chemical and non-chemical pesticides and output (yield) in the second cucumber experiment

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cultivar</th>
<th>Strategy</th>
<th># chem. sprays</th>
<th># PNO sprays</th>
<th>Yield (kg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Euphoria</td>
<td>Chem.</td>
<td>12</td>
<td>0</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biol.</td>
<td>3</td>
<td>10</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>Grendel</td>
<td>Chem.</td>
<td>2</td>
<td>0</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biol.</td>
<td>0</td>
<td>5</td>
<td>10.6</td>
</tr>
<tr>
<td>Art. light</td>
<td>Euphoria</td>
<td>Chem.</td>
<td>14</td>
<td>0</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biol.</td>
<td>5</td>
<td>9</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>Grendel</td>
<td>Chem.</td>
<td>4</td>
<td>0</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biol.</td>
<td>0</td>
<td>8</td>
<td>14.2</td>
</tr>
<tr>
<td>Humid</td>
<td>Euphoria</td>
<td>Chem.</td>
<td>10</td>
<td>0</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biol.</td>
<td>3</td>
<td>8</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>Grendel</td>
<td>Chem.</td>
<td>4</td>
<td>0</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biol.</td>
<td>0</td>
<td>7</td>
<td>11.8</td>
</tr>
</tbody>
</table>

Chrysanthemum: aphids were the most severe problem. No differences in the quality of the harvested product occurred between integrated and chemical control strategies, but the input of chemicals was lower in the integrated strategies. In the biological strategy, many flowers had to be discarded at harvest because of aphids (Table 2).
Table 2. Input of control measures and resulting scores on stems at harvest in the Chrysanthemum experiment

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Application of control compounds (#)</th>
<th>Cost of sprays + natural enemies (k€ per ha)</th>
<th>Environmental impact (see text)</th>
<th>Thrips score</th>
<th>Spider mite score</th>
<th>Stems with aphids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5</td>
<td></td>
<td></td>
<td>3.0 a</td>
<td>27.0 c</td>
<td>41</td>
</tr>
<tr>
<td>Chemical</td>
<td>18 chemical</td>
<td>3.8+0=3.8</td>
<td>476</td>
<td>3.3 a</td>
<td>22.8 bc</td>
<td>9</td>
</tr>
<tr>
<td>Biological</td>
<td>7 natural</td>
<td>1.2+3.0=4.2</td>
<td>56</td>
<td>1.5 a</td>
<td>15.8 ab</td>
<td>86</td>
</tr>
<tr>
<td>Integrated</td>
<td>13 chemical</td>
<td>1.7+3.0=4.7</td>
<td>47</td>
<td>2.5 a</td>
<td>21.3 bc</td>
<td>16</td>
</tr>
<tr>
<td>Integrated+ Nemasys F</td>
<td>8 chemical, 9</td>
<td>12.6+3.0=15.6</td>
<td>36</td>
<td>7.3 b</td>
<td>11.5 a</td>
<td>16</td>
</tr>
</tbody>
</table>

Numbers followed by the same letter are not significantly different at \( P \leq 0.05 \).

Table 3. Area Under the Disease Progress Curve (AUDPC) for powdery mildew in the rose experiment

<table>
<thead>
<tr>
<th>Strategy</th>
<th>AUDPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>369 a</td>
</tr>
<tr>
<td>Biocontrol + Natural products</td>
<td>237 bc</td>
</tr>
<tr>
<td>Chemical</td>
<td>216 c</td>
</tr>
<tr>
<td>Biocontrol</td>
<td>287 b</td>
</tr>
<tr>
<td>Natural products</td>
<td>247 bc</td>
</tr>
</tbody>
</table>

Numbers followed by the same letter are not significantly different at \( P \leq 0.05 \).

Rose: In the rose experiment, no differences in pests occurred between treatments. PM was the most severe problem. The presence of the untreated control caused a very high infection pressure in the glasshouse. All strategies significantly reduced PM (Table 3), but disease increased quite rapidly in all strategies. Still, the effect of the natural product KBV99.01 alone or in alternation with Sporodex was similar to chemical control (Table 3).

Discussion

In cucumber, artificial light enhanced yield but at the same time increased PM severity and should therefore be combined with partially resistant cultivars. Under high disease pressure in a susceptible cultivar, control of PM with a biocontrol agent and natural products was difficult in these experiments. Integration of these biological/natural products with chemical pesticides may improve control under these circumstances. The new natural product of Koppert B.V. appeared to be a promising new compound against PM and is compatible with Sporodex. Humid conditions can be chosen in autumn when there is a risk of Botrytis. However, both PM resistant cultivars and humid conditions increase the risk of infection by Mycosphaerella.

In chrysanthemum, integrated control was successful but more expensive than the chemical strategy. The ‘environmental impact score’ was much lower in the integrated strategies than in the chemical strategy. However, the chemical strategy included the use of Aaterra and was very risk-avoiding. A less conservative strategy would have decreased this score by 74% in the chemical control strategy. The use of Nemasys F increased costs but did not result in better control in this experiment, although it was effective in other experiments against thrips (Beerling, Applied Plant Research, The Netherlands, unpublished results). In chrysanthemum, pure biological control is not yet possible.
In rose, the different strategies resulted in similar pest control. PM was controlled to the same extent in strategies, which included the natural compound KBV99-01 as in the chemical strategy. Disease pressure was extremely high in this experiment and control would have been better without the presence of the untreated control.

In conclusion, well-chosen strategies, taking possible side effect on non-target pests into account, can result in similar control of pests and diseases as chemical control. The impact on the environment may be much lower, but this depends strongly on the components of the strategy. The strategies can include climate control and cultivar choice. Good scouting in the crop is important as it assures timely implementation of control measures.

**Implementation in practice**

In 2003, a new project was started to implement the most recent knowledge in practice. Four groups (tomato, cucumber, rose and Chrysanthemum) of 6-10 growers from different regions in The Netherlands have been formed. For the next several years, these growers are testing and implementing optimum control strategies, in collaboration with researchers and consultants from commercial companies. For each individual commercial glasshouse, the grower, researcher and consultant draw up a crop protection plan. All input in control measures and all output (quantity and quality of harvested product) are recorded. The results are analyzed for economic results and environmental impact and then the crop protection plan for the next crop is designed. Thus, the feasibility of new control strategies is tested. By organising excursions to the commercial glasshouses involved and by other means of communication, other growers will be encouraged to also implement the best new control strategies. The goal of the project is that most of the growers in the groups as well as 25% of the other growers use the advised control strategies at the end of the project in 2006.

**Acknowledgement**

The work was funded by the Dutch Ministry of Agriculture, Nature Management and Food.

**References**

Verticillium lecanii (Lecanicillium spp.) as epiphyte and its application to biological control of arthropod pests and diseases

Masanori Koike1, Toshiki Higashio1, Akio Komori1, Kyouko Akiyama1, Noriko Kishimoto1, Emi Masuda1, Mai Sasaki1, Sanae Yoshida1, Masayuki Tani1, Katsuhiko Kuramoti1, Midori Sugimoto2, Hideyuki Nagao3

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Abstract: Twenty strains of Verticillium lecanii (Lecanicillium spp.) isolated from plant pathogenic fungi, aphids, and whiteflies, including two commercialized strains (Mycotal and Vertalec) were tested for their abilities to colonize the leaf surfaces of four plant species (cucumber, strawberry, tomato, and wheat) grown in a glasshouse. Leaf surfaces were inoculated with V. lecanii by spraying conidia suspensions (1 × 10⁷/ml/leaf) of each strain. We confirmed the abilities of colonization on leaf surfaces by dilution plate method. Three strains (A-2, B-2, and C-1) showed higher ability of colonization at 2 and 4 week after inoculation compared to other strains. These strains were studied to determine their potential as an epiphytic biocontrol of whitefly (on cucumber and tomato), aphids (on tomato) and powdery mildew (on cucumber and melon). Data from glasshouse studies indicated that these three epiphytic strains, especially B-2 inoculation to leaves of tomato and cucumber, reduced not only the population growth of whitefly (on tomato, cucumber and melon) and aphids (on tomato, cucumber and melon), but also disease symptoms of powdery mildew on cucumber and melon, and strawberry grey mould. Therefore, B-2 may be a better candidate to advance toward development as a microbial control agent.

Key words: aphids, disease, epiphyte, Verticillium lecanii, pests, whitefly

Introduction

Entomopathogenic fungus Verticillium lecanii (Zimm.) Viégas is known to attack parasites such as aphids and whiteflies (Hall, 1984). Several reports have demonstrated the pathogenicity of V. lecanii toward aphids and whiteflies under laboratory and greenhouse conditions. Vertalec- and Myco tal-formulated spores of V. lecanii were marketed for aphids and whitefly control in greenhouses, respectively. V. lecanii may also be an effective hyperparasite of several rust fungi, including Uromyces dianthi on carnation (Spencer, 1980), U. appendiculatus on dwarf bean (Grabski & Mendgen, 1986), and Puccinia recondite on wheat (Spencer & Atkey, 1981), as well as of some powdery mildew pathogens, such as Oidium tingtanium on citrus (Raghavendra & Pavgi, 1977), Erysiphe graminis on barley (Hall, 1980), and Sphaerotheca fuliginea on cucumber (Spencer & Ebben, 1981; Verhaar et al., 1996). The objectives of this study were to: (1) evaluate epiphytic ability of V. lecanii and select epiphytic strains as candidates of biological control agents; (2) investigate the potential of epiphytic V. lecanii as biological control agents for plant pests and pathogenic fungi.
Material and methods

Fungal isolates
Twenty isolates of *V. lecanii* were used in this study. Eighteen out of 20 isolates originated in Japan, EU countries and United States. Commercial preparations of Mycotal and Vertalec from Koppert UK Ltd. (Wadhurst, East Sussex, UK) were also included. Isolates of *V. lecanii* were cultured on potato sucrose agar (PSA: 200 g of potato, 20 g of sucrose, 20 g of agar per liter) or broth containing chitin as the source of carbon and nitrogen. Conidia were harvested from 10-day-old cultures by flooding the plates with distilled water, or after 1 week of incubation from the liquid medium by filtering through cheesecloth. Suspensions were adjusted to the desired density using a haemocytometer. Surfactant (one drop of Tween 80 per 20 ml) was added to the suspension by 20 ml per liter.

Glasshouse condition and plants
Plants of cucumber cv. ‘Tokiwa Hikari No.3 P’, strawberry cv. ‘Meho’, tomato cv. ‘Momotaro’, wheat cv. ‘Hokushin’ and melon cv. ‘Red113’, were grown in climate-controlled glasshouse at 25±1ºC (day) and 20±1ºC (night), and relative humidity was 40% (day) and 60% (night).

Colonization on the leaf surface
The 20 isolates of *V. lecanii* were used for examinations. Conidia concentrations were adjusted to 1 × 10⁷ spores/ml. For colonization tests, suspensions of *V. lecanii* were sprayed on second to fourth true leaf surfaces of cucumber, strawberry, tomato, and wheat plants (c. four weeks after germination) using a customized sprayer. Epiphytic abilities were estimated using dilution plate method.

Control of whitefly, aphids and disease in a glasshouse and a field
We used A-2, B-2, C-1, Vertalec, and Mycotal to control insects and disease. *V. lecanii* were sprayed bi-weekly in a glasshouse (cucumber, tomato, and melon), a greenhouse (cucumber, tomato and melon), and a field (strawberry). The population of whitefly and aphids (green peach aphid and cotton aphid) were estimated using the direct count method on 3–5 plants per plot. Disease (grey mould of strawberry, and powdery mildew of cucumber and melon) incidence also counted.

Results and discussion

Colonization on the leaf
Table 1 summarizes colonization on the leaf. In these studies, A-2, B-2, and C-1 showed high colonizing ability on plant leaf surfaces, whereas commercial preparations of Mycotal and Vertalec did not.

Table 1. Epiphytic ability of *V. lecanii* on the plant leaf

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Cucumber (cfu)</th>
<th>Tomato (cfu)</th>
<th>Strawberry (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-2</td>
<td>5.0 × 10⁴</td>
<td>4.9 × 10³</td>
<td>6.4 × 10²</td>
</tr>
<tr>
<td>B-2</td>
<td>2.9 × 10⁴</td>
<td>1.5 × 10³</td>
<td>2.0 × 10³</td>
</tr>
<tr>
<td>C-1</td>
<td>1.4 × 10⁴</td>
<td>3.4 × 10³</td>
<td>2.2 × 10³</td>
</tr>
<tr>
<td>Vertalec</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mycotal</td>
<td>3.3 × 10⁴</td>
<td>6.2 × 10⁴</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cfu were estimated per cm², 4 weeks after treatment. ND: not detected.
**Control of insects and diseases using V. lecanii**

We found A-2, B-2 (*V. dahliae* epiphytic strains) to be effective for population control of whiteflies and aphids on cucumber, tomato, and melon, and for disease control of powdery mildew of cucumber leaf. Nevertheless, they were ineffective on melon powdery mildew in a controlled glasshouse and an uncontrolled greenhouse. Table 2 shows effects of *V. lecanii* for control of insects and diseases in a glasshouse, a greenhouse, and a field.

A field trial demonstrated that A-2, B-2 and Vertalec had an effect on controlling aphid population. There were no effects on untargeted insects (data not shown). Only B-2 treatment decreased the disease incidence of grey mould on strawberry fruits (data not shown).

### Table 2. Effect of *V. lecanii* for control of whitefly, aphids and disease in a controlled glasshouse and an uncontrolled greenhouse

<table>
<thead>
<tr>
<th>Plant Isolates</th>
<th>Cucumber</th>
<th>Melon</th>
<th>Tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Powdery mildew</td>
<td>Whitefly</td>
<td>Aphids</td>
</tr>
<tr>
<td>A-2</td>
<td>+++ ++</td>
<td>+++ ++</td>
<td>++ ++</td>
</tr>
<tr>
<td>B-2</td>
<td>++ +++</td>
<td>+++ ++</td>
<td>+++ +++</td>
</tr>
<tr>
<td>C-1</td>
<td>++ NT</td>
<td>± NT</td>
<td>+ NT</td>
</tr>
<tr>
<td>Vertalec</td>
<td>– –</td>
<td>– –</td>
<td>+ –</td>
</tr>
<tr>
<td>Mycotal</td>
<td>– –</td>
<td>++ ++</td>
<td>± –</td>
</tr>
</tbody>
</table>

* In each cell, the left row shows the effect in a controlled glasshouse and the right row shows the effect in an uncontrolled greenhouse (–, no effect; ±, unstable effect; +, < 80% of insect population and disease incidence compared with the control treatment; ++, < 60%; ++++, < 40%). NT: not tested.

### Table 3. Multiple effects of entomopathogenic *Verticillium lecanii*

<table>
<thead>
<tr>
<th>Isolates</th>
<th>A-2</th>
<th>B-2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogenicity</td>
<td>Aphids</td>
<td>Aphids</td>
<td></td>
</tr>
<tr>
<td>Whitefly</td>
<td><em>Botrytis cinerea</em></td>
<td><em>Botrytis cinerea</em></td>
<td></td>
</tr>
<tr>
<td>In vitro antagonistic to</td>
<td><em>Verticillium dahliae</em></td>
<td><em>Verticillium dahliae</em></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td><em>Fusarium oxysporum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease in disease incidence</td>
<td>Powdery mildew (cucumber and melon)</td>
<td>Powdery mildew (cucumber and melon)</td>
<td></td>
</tr>
<tr>
<td>Grey mould (strawberry)</td>
<td>Grey mould (strawberry)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium wilt (melon)</td>
<td>Fusarium wilt (tomato)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Commercialised strains of *V. lecanii* – Mycotal and Vertalec – require high humidity (>97%) to obtain good control. Humid conditions on plant surfaces are probably the most important factor influencing the germination, growth and survival of *V. lecanii* in this habitat (Verhaar et al., 1996). Whereas A-2, B-2, and C-1 marked high colonizing ability under low relative humidity (about 40%-60%) conditions during 4 wk following the application. This ability suggests that these three strains, especially strain B-2, may be better candidates for biocontrol.
agents than the other strains of *V. lecanii* on commercialised glasshouse-grown crops. Table 3 also listed multiple effects of *V. lecanii* strains A-2 and B-2.

**Acknowledgements**

We thank Maruwa Biochemical Co., Ltd. and Pioneer EcoScience Co Japan for financial support.

**References**


Integrated Pest Management of arthropod pests in stone fruits

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Abstract: Pest management in fresh market stone fruit production in the United States has relied on organophosphate and carbamate insecticides to reduce arthropod pest damage since the development of these pesticides in the 1940’s. Although quite effective, environmental problems and consumer concerns have arisen. Beginning in 2000, a program to manage arthropod pests in peach, nectarine, and plum without these pesticides was initiated. This program relied on mating disruption for Oriental fruit moth Grapholitha molesta, horticultural mineral oil application for San Jose scale Diaspidiotus pernicius and European red mite Panonychus ulmi, bloom time applications of Bacillus thuringiensis or spinosad for peach twig borer Anarsia lineatella, and western flower thrips, Frankliniella occidentalis, and summer sprays of spinosad for forkedtailed bush katydid Scudderia furcata and omnivorous leafroller Platynota stultana. Ten farms were selected whereby orchards of the same variety compared the above management approach (Reduced Risk) with the Conventional approach. The Conventional approach utilized the standard organophosphate and carbamate pesticides for managing key pests. The orchards were intensively monitored each week from February through September. Orchards were located in five peach producing counties in California’s San Joaquin Valley. The results of the study showed no significant (P≤0.05, Fisher’s Protected LSD) difference in pest damage and no difference in grower costs between the two approaches to pest management. The four-year average of insect damage for the Conventional and the Reduced Risk orchards was 5.58% and 5.83% respectively. Key pests such as Oriental fruit moth, San Jose scale, and peach twig borer averaged less than 1% each year. The greatest damage occurred from forkedtail bush katydid, a recently recognized pest, and western flower thrips. The four-year average katydid damage was 3.25% and 3.21% for the Conventional and Reduced Risk Programs. The cost per acre averaged $190 for each program.

Key words: Integrated pest management, peaches, Oriental fruit moth, San Jose scale, forkedtail bush katydid, IPM implementation

Introduction

In 2000 California peach and plum farmers, University of California entomologist and pomologists, California Department of Pesticide Regulation personnel, Pest Management Consultants, The Environmental Protection Agency (Region 9) and Fresno State University pomologist formed a group called the Stone Fruit Pest Management Alliance. It was developed to implement pest management programs that do not rely on pesticides that are likely to be eliminated in the United States by the Food Quality Protection Act of 1996, because of surface water contamination due to dormant spray runoff or because of consumer concern over pesticide residues in food. The project emphasized monitoring and knowledge of pest and beneficial arthropod biology to determine when to use strategies such as mating disruption, dormant oil sprays, and non-disruptive pesticides and to substitute these environmentally friendly practices for the more conventional broad-spectrum pesticides. The goal was to develop and demonstrate programs with equal or less pest damage and pesticide cost. Reducing reliance on organophosphate and carbamate pesticides will lessen the likelihood of...
banning them from use. Such pesticides are important for managing plant bugs and spotted cucumber beetle where other materials are not effective.

Materials and methods

The project was designed so that each cooperator would manage two blocks of the same variety of fruit. One block would utilize the more traditional pesticides such as chlorpyrifos, diazinon, phosmet, carbaryl, methomyl, and methidathion, when needed. This traditional management comparison is titled “Conventional”. The other block would be managed with pheromone mating disruption, horticultural mineral oils, Bacillus thuringiensis, and spinosad, when needed. The second style of pest management is titled PMA. The decision on using pesticides is based upon the University of California Integrated Pest Management Guidelines.

Monitoring is a key part of the Pest Management Alliance Program. Knowing the status of pests and beneficial arthropods both inside and outside the orchard can be a guide in deciding upon the use and type of insecticide. The information gained from this detailed monitoring allows for analysis of data gaps in detecting pest damage. Information on pests and beneficial arthropods was tabulated. The following monitoring guidelines were in place during the season.

Dormant season
Scaffold twigs were searched for live San Jose scale, Diaspidotus perniciosus (SJS), and European red mite, Panonychus ulmi (ERM). The sample unit is the proximal 7.5 cm of shoots arising from the inner portion of the scaffold and these are examined under a microscope. Fifty such shoots are sampled from both the Conventional and the PMA blocks. Five twigs are taken from each of ten trees per block.

Early bloom
Oriental fruit moth (OFM), SJS and omnivorous leafroller (OLR) pheromone traps were placed in each block. Two traps per block were used. Assessment of adult western flower thrips (WFT) in flowers utilized beating tray samples and flower examination. A single shoot per tree (each shoot having 10 flowers), and fifty trees per block are sampled. A “beat” is three abrupt raps of the flowering shoot over the tray.

Late bloom
San Jose scale crawlers were sampled in orchards using double-sided sticky tape. The sample unit is an individual tape around a branch at least 1 inch in circumference. Tapes are placed on 10 trees per block. Flower dissections were accomplished after sampling fifty flowers per block.

Petal-fall
Forktailed bush katydid, FTBK, egg/nymph sampling was done. This involves sweeping of the cover with a sweep net for nymphs and examining the lower scaffold area of 50 trees for 1 minute to detect leaf feeding. Two PTB traps were placed per orchard. Twenty-five trees per block were monitored for OFM by counting the number of flagged shoots. San Jose scale sticky tapes examination was continued. Sweep net samples of 50 sweeps per block for lygus bugs and stinkbugs were continued.

Spring and summer
Fifty fruit were examined for signs of thrips, worms, or katydid damage in alternate weeks prior to harvest. Spider mites were sampled by collecting leaves. Ten leaves from each of five trees are sampled and counted. The number of mites was not tabulated, but the proportion of infested leaves was tabulated. An action threshold of thirty percent infested leaves was used. Harvest samples of 500 to 1,000 fruit were sampled as harvest occurs. This is done prior to sorting by harvesting crew.
Arthropod counts were tabulated on a weekly basis and also as a seasonal total on a per trap basis for each block monitored. Total arthropod counts were compared between the PMA and the Conventional orchards. Insect damage counts were also taken every other week and at harvest. Costs of each pest management approach (materials but not application) were tabulated and presented.

**Information dissemination**

Information dissemination occurred through field meetings, mailings, and a Tree Fruit Pest Management web site (http://www.uckac.edu/treefruitipm/). In 2002, a CD ROM was created which described the results of the study and was distributed to farmers. Finally a public television program entitled “American Environmental Review” was produced.

**Results and discussion**

The Alliance generated substantial information on reduced-risk pest management efficacy and cost. Insights were gained as to how well the various management techniques worked within actual commercial stone fruit production systems. The 4-year average per acre cost of pesticides in the PMA managed orchards was $189.75 (SE±$14.82). The same 4-year average cost in the Conventionally managed orchards was $189.25 (SE± $12.94). Table 1 presents the annual costs. The average percent damage due to arthropods was 5.88 (SE± 1.52) and 5.60 (SE± 1.29) for The PMA and Conventional orchards respectively. Table 2 presents the average pest damage.

The two most damaging arthropod pests were forktailed bush katydid, *Scudderia furcata* and western flower thrips, *Frankliniella occidentalis*. No damage was found due to peach twig borer, *Anarsia lineatella*. The two primary pests of greatest concern when the program was started were Oriental fruit moth, *Grapholitha molesta* and San Jose scale *Diaspidiotus perniciocus*. Only minor damage was attributed to these latter two pests throughout the program.

Based on the results of this four-year study, stone fruit farmers can effectively manage arthropod pests with reduced risk pesticides. The damage and costs of such a program is not different than the more widely used programs that emphasize organophosphates and carbamates.

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>218</td>
<td>148</td>
<td>198</td>
<td>195</td>
</tr>
<tr>
<td>Conventional</td>
<td>221</td>
<td>159</td>
<td>182</td>
<td>195</td>
</tr>
</tbody>
</table>

During the four years of the Stone Fruit Pest Management Alliance, 26 orchard comparisons were made. These included 16 nectarine orchards, 8 peach orchards, and 2 plum orchards. Eight stone fruit farmers were involved and the farms were located in five southern San Joaquin Valley Counties. Harvest dates ranged from May 3 to September 20. In general terms, nectarines pose the greatest pest management challenge, followed by peach and then plum. However, harvest date will also have a bearing. Nectarines harvested in May pose less of a challenge than plums harvested in September.
Thirty-nine presentations were made in Kern, Tulare, Kings, Fresno, Stanislaus, Madera, Merced, San Joaquin, Sutter/Yuba and Sacramento County. Over 1,200 individuals attended these presentations.

Table 2. Annual damage (%) in Pest Management Alliance and Conventionally managed orchard comparisons

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>5.7</td>
<td>6.7</td>
<td>9.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Conventional</td>
<td>5.6</td>
<td>6.5</td>
<td>8.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Acknowledgements

The authors would like to thank the cooperating growers. They are John Deniz, Robert Jackson, Ron Metzler, Pat Pinkham, Steve Strong, Rick Schellenberg, John Kovacevich, and Bill Tos. Pest control advisors contributing to this study included Les Nygren, Keith Heinricks, and Judy Stewart Leslie.

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Biocontrol agents and their integration in organic viticulture in Trentino, Italy: characteristics and constrains

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Abstract: The use of biocontrol agents in organic pest management and the present situation of organic viticulture in Trentino have been documented with a survey done in the collaboration with the Organic Growers Association of Trentino (ATABIO). The objective was to evaluate the incidence and severity of diseases in biological grown vineyards and the existing problems and constrains in the non-chemical control of grapevine pests. Downy mildew was found to be the main problem, followed by powdery mildew and mites. Grey mould is a problem only in extremely wet areas, but can be managed with agronomical techniques. Weeds are a serious problem during the early stages of new vineyards. In general pest and diseases are not severe constrains for organic growers in Trentino and they are mainly controlled with sulphur, copper, pyrethrum and mating disruption. BCAs would be more frequently applied if information on their application and efficacy will be available from valid experimentation.

Keywords: organic viticulture, BCAs, pest management, Trentino

Introduction

Organic agriculture is a thriving sector in Europe, and in some countries 10% of the agricultural area is organic. However in viticulture percentage of organic vineyards is lower than the average for all organic agriculture. This is due to several constraints: in the sector of production techniques, in marketing, in legislation and public support (Willer et al., 2002). Demand for organic products is continuously increasing in Italy and Europe. In Trentino region, organic viticulture is also increasing, responding to the market requests.

Organic viticulture aims at improving products (wine and table grape) quality by avoiding risks for health and environment that are due to chemical pest management. Organic viticulture has trigged off several innovations also in conventional viticulture. Organic farmers promote “biodiversity” and allow plants other than vines to grow in and around the vineyard. Biodiversity helps regulate the vineyard soil by attracting beneficial insects, spiders and predatory mites, as well as provide shelter and food (pollen, nectar and other bugs), and replaces the need for chemical pesticides or insecticides. What cannot be fully controlled through biodiversity can mostly be managed organically, through the use of naturally occurring plant or mineral extracts, which leave no residues in the soil, however the massive use of copper is under question.

Pest control in organic agriculture has to be managed with a holistic approach, and biocontrol agents (BCAs) could be a useful tool both against disease and insects. The use of BCAs in organic pest management and the present situation of organic viticulture in Trentino have been documented with a survey done in collaboration with the Organic growers
association of Trentino (ATABIO). The objective was to evaluate the incidence and severity of diseases in biologic vineyards and the existing problems and constrains in the non-chemical control of grapevine pests.

**Results and discussion**

In the Trentino Province (North-eastern Italy), the surface of the organic viticulture shows a continuous increase in the last ten years (Figure 1). In 1995 less than 10 ha of the vineyards were managed organically, nowadays this surface is about 60 ha, whereas the conventional/integrated covers ca 9,500 ha.

![Figure 1. Evolution of the organic viticulture surfaces grown in Trentino](image)

In this survey we interviewed 25 organic farmers, whose vineyards were managed organically; these farms represent about the 95% of the total organic viticulture in Trentino and their crop surface is about 100 ha. Only three of the checked farms exclusively cultivate grapevine (Figure 2). In the other farms, apple, small fruits, horticultural crops and other minor crops (chestnut, plum, olive etc.) are also cultivated.

![Figure 2. Percentages of different crops grown in the organic farms in Trentino (surface), total surface: 100 ha](image)
Considering only the organic vineyards, the total surface is about 50 hectares with 2.5 ha of average surface per farm. About 20 grapevine cultivars are cultivated, but only ten of them have a cropped surface higher than 2 ha (Figure 3); the most common cultivars are Chardonnay and Merlot followed by Cabernet Sauvignon, Pinot noir and Mueller Thurgau; few other minor and local varieties (Nosiola, Rebo, Lugliatica, Incrocio Manzoni etc.) are also present, but mainly in new plantations. Downy mildew resistant varieties are not present.

Regarding the final destination of the organic grape, only 40% of farms produce wine by themselves; remaining organic winegrowers deliver the grapes to different cooperative wineries. One challenge for organic wine producers remains communicating to consumers that organic wines can reach the high quality of conventional/IPM ones. In relation to wine, the term “organic” is a difficult concept to define and there is a lack in legislation. The term “organic wine” cannot be used in Italy, but on the label it can be only written that “the wine is made from organic grown berries”, which means that grapes have been grown and processed without the use of synthetic fertilisers, chemical herbicides, chemical disease-control sprays, insecticides, growth regulators, flavour-enhancers or genetic engineering. Major marketing efforts are therefore needed.

Concerning the organic vineyard pest and disease management, downy mildew represents the main problem, followed by powdery mildew and mites. Gray mould is a problem only in specific areas with high humidity, but can be managed with agronomical techniques. BCAs (Trichoderma harzianum, T. viride, Ampelomyces quisqualis) are not commonly used and disease control is usually achieved with copper and sulphur (10-17 treatments in total, with an average of 10-12), mainly because these treatments are considered easier to apply, cheap, reliable and effective. Mating disruption is used to control vine moths. There is an increasing worry about the possible diffusion of Flavescence doréé from the near region (Veneto) and treatments against Scaphoidaeus titans are done with pyrethrum, where this vector is present. BCAs against insects (Bacillus thuringiensis, Beauveria bassiana) are, similarly to diseases, scarcely applied for the same reasons.

The most challenging aspect of growing grapes organically is weed control, because it can be done only via mechanical means. For this reason weeds are a big problem especially in
the early stages of new vineyards; moreover the weed management is very expensive too, because of the steep slope of several vineyards.

Growers are generally satisfied about public extension service, even if there is a common demand for more attention to organic farms. The information on diseases and pest management is derived mainly from books and specialised papers. Other growers or private consultants are not an important source of information. Ideological motivations or personal decision guided farmers to start an organic farm or convert it from IPM. Some made the choice to cut out synthetic materials because of illness in the family or out of a desire to make the farm a safer place for their children. Only for few farmers economics are the primary motivation.

In conclusion, apart some problems at the beginning of a new plantation or during the conversion from IPM to organic management, pest and diseases are not severe constrains for organic growers in Trentino and they are mainly controlled with sulphur, copper, pyrethrum and mating disruption. BCAs would be more frequently applied if information on their application, reliability and efficacy will be available from valid experimentation.

Acknowledgements

The authors kindly acknowledge M. Scrinzi (ATABIO), G. Visintainer (Province of Trento) and all the growers for their collaboration in this study. The research was supported by Fondo per la ricerca, Provincia Autonoma di Trento, AGRIBIO project.

References

Screening of microorganisms and other alternative seed treatments for activity against seed-borne pathogens of cereals

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Historically, seed-borne pathogens have been one of the major obstacles in cereal production. In conventional agriculture where highly effective chemical seed treatments are regularly used, seed-borne pathogens are nowadays considered only a minor problem. However, in organic farming seed-borne pathogens are apparently becoming increasingly important. The main reason is the lack of effective methods of seed treatment allowed to be used in organic farming. We performed a screening under controlled conditions for activity against common bunt (*Tilletia caries*) of wheat with laboratory preparations of microorganisms belonging to different taxa (*Trichoderma*, streptomycetes and other bacteria), formulated microbial products, plant extracts and resistance inducing agents. Treatments with activity were observed in each of these groups, except in the group of resistance inducing agents. When effective treatments were repeatedly tested, a large variability was observed between the experiments. This was especially true in case of the bacteria, but less pronounced for the tested isolates of *Trichoderma* and *Streptomyces*. The most active treatments were included in a field trial and further evaluated in greenhouse tests for activity against other important cereal seed-borne pathogens (leaf stripe of barley, *Helminthosporium gramineum*; net blotch, *Drechslera teres*; *Fusarium*-seedling blight). The results will be presented and discussed.
Seed treatments for organic vegetable production

Annegret Schmitt, Eckhard Koch

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Due to the difficulty in organic farming of producing pathogen free seeds, and the lack of simple, effective non-chemical methods for seed sanitation, a substantial part of the seed used by European organic vegetable growers is still derived from conventional production. This will be strongly restricted after the year 2003 (EU Council regulation 2092/91). In March 2003, a EU-project "Seed Treatments for Organic Vegetable Production" (QLRT-2002-02239; STOVE) was initiated. The project is aimed at improving currently available, non-chemical methods for control of seed-borne vegetable pathogens and to develop new methods, which are acceptable to organic farming. The participants are Federal Biological Research Centre for Agriculture and Forestry (BBA), Germany (M. Jahn, E. Koch, C. Kromphardt, A. Schmitt); Plant Research International (PRI), Wageningen, Netherlands (S. Groot, J. v.d. Wolf); Swedish University of Agricultural Sciences (SLU), Uppsala, Schweden (G. Forsberg, B. Gerhardson); University of Gothenburg, Sweden (T. Amein, S. Wright) - Findus R&D AB, Bjuv, Sweden (R. Stegmark, M. Wikström); Nunhems Zaden BV, Haelen, Netherlands (J. v.d. Berg); Nunhems Zaden (Hild), Marbach, Germany (M. Mistele, S. Werner); University of Turin, (Agrinnova) Italy (M. Gullino, F. Tinivella); Horticulture Research International (HRI), Wellesbourne, Great Britain (S. Roberts). Together with three physical methods (hot water, hot air and electron treatment), microorganisms and other agents of natural origin acceptable to organic farming will be included in the project. Initially, the three physical methods are being adapted for different vegetable species (e.g. carrot, parsley, cabbage, lamb’s lettuce, basil, bean) and their respective seed-borne pathogens (e.g. A. dauci, S. petroselini, X. campestris, P. valerianellae, Fusarium spp., C. lindemuthianum). In parallel, potential alternative seed treatments (micro-organisms, plant extracts, inducers of resistance) are also being tested. The efficacy of the methods will be compared in glasshouse and field trials, and selected combinations will be evaluated. Special regard will be placed on physiological factors determining the sensitivity of seeds towards the physical methods. The results of the first experiments were presented. (See http://www.stove-project.net/ for further information).
Implementation of IPM strategies on greenhouse tomato on Oeste region of Portugal: case study

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Abstract: Since the eighties production of protected vegetable crops has increased in Oeste region of Portugal, particularly tomato crop that represents 52% of the total area and 50% of the total national production (Mexia, 1990; INE, 2003). The aim of this study conducted in a modern greenhouse on rockwool and coconut fibre was to follow up the tomato crop under IPM. For this purpose observations were carried out weekly and the diseases and pest species complex were identified as well as the entomophagous species present. The experimental work was designed in order to validate an IPM programme (risk assessment methodologies, decision tools) previously set for Oeste region – the PAMAF model (Marques et al., 1999). The results of this IPM model validation are presented concerning risk assessment evaluation, decision tools concerning more specifically whitefly problems. The validation of these risk assessment techniques for the key-pests revealed major differences in the whiteflies’ case. Statistical differences between this method and the entire plant observation were found for adults and, at a much smaller level, for nymphs. However, observations on three randomly picked leaflets from the medium plant level led to the same control decision as observations of the entire plant and so the less time consuming PAMAF model is accepted for nymphs risk assessment. The establishment of a sample size of 30 units was not possible for all cases of incidence levels.

Key words: IPM, tomato crop, whiteflies, risk assessment

Introduction

During the end of nineties SAPI/DPPF from Instituto Superior de Agronomia in Lisbon developed experimental work on IPM in greenhouse vegetable crops, namely tomato, green beans and lettuce. The major goal was the definition of a risk assessment methodology and decision rules in order to support an IPM program to the Portuguese Oeste region. The experimental program was based on national experience, literature and experiences reported in Europe, especially on the research developed around IOBC/WPRS Working Group on Integrated Control on Protected Crops, Mediterranean Climate. Three years of field work has built up regional data and in 1999 the results were published and a handbook was disseminated to the growers (Marques et al., 1999).

In sequence a new project with a strong demonstrative component, supported by an agricultural operational program (PO-AGRO, nº4), in progress, established the target to confirm such proposed methodologies. An important purpose of the crop protection research in this project is to develop “easy-to-use” risk assessment methodologies. Recent studies demonstrated that the lack of friendly methods on which to base decision-making is one of the most important constraints for IPM practice (Rodrigo et al., 2000).
In particular the methods can be improved in two time consuming ways: decreasing sample size or defining smaller sample units.

In this work a study carried out during the 2003 tomato-growing season (March-August) is presented. Whiteflies (*Trialeurodes vaporariorum* Westwood) are one of the major pest problems and imply huge indecisions about risk assessment, especially the definition of the observed stage of the pest, sample unit and optimum sample size as well as decision rules related with control methods. Other pest problems and diseases detected are also presented as well as the solutions adopted in IPM.

**Material and methods**

**Greenhouse**

The data in this study resulted from weekly visual observations in a modern greenhouse of 3000 m². An experiment was designed to test different varieties concerning production and organoleptic aspects. To test the IPM model, 96 plants were weekly randomised and observed. The visual observation of each plant was done following two schemes. The first scheme was based on the methods performed for each pest by Marques et al. (1999) (PAMAF method). According to these authors the method to detect whiteflies (adults and nymphs) is the examination of three leaflets on the top of the plants (above the upper flower cluster). In the second scheme whole plants were observed (all leaves looking for any stage of each pest) (Entire Plant method). In the present work the exercise to confirm methods for risk assessment for whiteflies is presented, specifically visual methods.

**Data analysis**

Data was changed to binomial variable: present/not present (0-1). First, data collected from the two methods were compared computing the difference between the values, and analysing the histogram (0-1). For whiteflies larva and adults data were compared by the non-parametric McNemar test on each date sample. This statistic analysis was supported by SPSS software (10.0 version). To study the possibility of decreasing the sample size to 30 units, binomial probabilities were calculated (Reis et al., 2001) for different whitefly population density traduced by incidence degree.

**Results and discussion**

**Key-pests and diseases**

*Botrytis cinerea* and *Leveillula taurica* were the most important airborne diseases and *Fusarium oxysporum f.sp. lycopersici* was the problem which limited production. The key pests were whiteflies, *Trialeurodes vaporariorum* (Westwood) and fruitworms, *Helicoverpa armigera* (Hbn). Towards the end of the growing season *Aculops lycopersici* (Massee) were found attacking the crop and was the main pest problem constraining the crop cycle to finish. Mirids, *Coenosia attenuata* Stein and *Hyposoter didymator* Thunberg were the most common entomophagous species reported.

**PAMAF model validation for key-pests**

The analysis related with model validation issues using descriptive methods evidenced that the major difference between the two described methods were on whiteflies data. For the other key-pests (aphids, caterpillars, leafminers, spider mites and thrips) data didn’t reveal important differences between the two methods.
Comparison of the 96 sample units: PAMAF method versus Entire Plant observation method for whiteflies

A significant number of sample units with whitefly adults revealed statistical differences, especially for adults. The PAMAF method was significantly less able to find the adults on the plants. This means that a significant number of *T. vaporariorum* adults were not located on the top of the plants despite of what is commonly reported in literature. Nevertheless, whitefly adults are easily found on yellow adhesive traps. On the other hand, data about nymphs’ detection only revealed two cases with statistical differences. In spite of this the decisions made with the two methods were not altered by these results (Table 1). The PAMAF method was efficient in detecting nymphs on the plants; so, it is sufficient to look for nymphs on three randomised leaflets from the medium plant level.

Table 1. Comparison of two risk assessment methods for whiteflies: PAMAF and entire plant

<table>
<thead>
<tr>
<th>Nº of tie cases (max. 24 dates x 96 plants)</th>
<th>Nº of divergent cases</th>
<th>Nº of studies with significant differences (McNemar test P&lt; 0.05)</th>
<th>Nº cases of different decision-making</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nymphs</td>
<td>2,292</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Adults</td>
<td>1,942</td>
<td>362</td>
<td>16</td>
</tr>
</tbody>
</table>

Testing the sample of 30 units

One of the major goal is to guarantee that a small and practical sample done by routine by the growers is enough to take decisions (chemical control or biotechnical control) avoiding spraying every week. In the project samples of 96 plants were done and that information was the base to make decisions. A test was done to find out if a sample size of 30 would lead to similar results. Firstly, an evaluation of the distribution type – $s^2/m$ – was made and confirmed that the result was inferior to 1, which implied the adoption of a binomial distribution. Secondly, the probabilities of occurrence according with the binomial distribution were computed by simulation with the possibilities of incidence (presence/not presence) on the 96 plants sample:

$$p = \binom{30}{x} \left(\frac{x}{96}\right)^x \left[1-(\frac{x}{96})\right]^{(30-x)}$$

and an economic threshold of 30% of incidence.

In this study the error levels accepted were:

- type I (treat without need) ≤ 0.20; 
- type II (need to treat and decide not to) ≤ 0.05;

The results revealed that sample size of 30 units (three leaflets on the medium plant level) was only not acceptable when whitefly incidence was between 25% and 50%.

Conclusions

Incidence of aphids, caterpillars, leafminers, spider mites and thrips present in a tomato crop can be assessed by the sample units defined by Marques et al. (1999). However sample size was not validated. Incidence of whitefly can be assessed by observing nymphs on the sample unit defined by Marques et al. (1999) (three leaflets on medium plant level) which is much
less time consuming than observing whole plants. Whitefly adults of *T. vaporariorum* are not only present at the top of the plant, in a significant frequency. Sample size is dependent of the population density. For incidences below 25% and above 50% a sample size of 30 units is enough for decision-making. Future studies including data collected during 2004 will allow the definition of a sequential sampling method.

**Acknowledgement**

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**References**


An integrated approach to simultaneously control insect pests, powdery mildew and seed borne fungal diseases in barley by bacterial seed treatment

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Abstract: In connection with extensive testing of a large number of bacteria for biological control of fungal seed borne pathogens, we have noted on several occasions that application of these bacteria to seeds also protects against insect pests and diseases attacking aerial plant parts. Thus a single treatment can offer the plants protection against a large variety of agents. The method of applying bacteria to seeds of cereals has been developed for large scale commercial use by BioAgri AB (Uppsala, Sweden), for example in the product Cedomon™. Twenty eight diverse bacterial strains representing diverse taxa isolated from different Swedish and Moroccan soils were tested for induction of resistance to powdery mildew in barley (Blumeria graminis f. sp. hordei). Seeds coated with bacterial culture broth resulted in plants that were either more susceptible or more resistant to powdery mildew. The four strains that induced the highest level of resistance to powdery mildew were tested more stringently in repeated tests. They were also tested for protecting barley plants from visitation of leafhoppers (Psammotettix alienus). A pot containing plants that had emerged from untreated seeds was placed in the same insect cage as one that contained plants that had emerged from treated seeds. The pots had no physical contact, even through irrigation water. Several leafhoppers were placed in each cage when the plants were 10 days old. The feeding preference of the leafhoppers toward plants emerging from treated vs. untreated seeds was recorded over a 14 day period. The results from repeated greenhouse trials indicate that certain strains of bacteria have a great potential not only to control seed borne diseases of cereals, but also to induce resistance to leafhoppers and powdery mildew, whereas others only induce resistance to leafhoppers. The diversity in the response of plants to bacterial seed treatment is intriguing. Ongoing field trials are assessing the persistence of this resistance response under field conditions. The most effective strains will be candidates for commercial development of multipurpose biocontrol agents.

Key words: induced resistance, biological control, Pseudomonas

Introduction

There is a large number of microorganisms in the rhizosphere and phyllosphere of plants whose importance to the ecology, growth and condition of plants is not very well known (Gerhardson & Wright, 2002). Some of them can induce enhanced plant growth and resistance to diseases caused by pathogenic bacteria or fungi (Kloepper et al., 1993; Van Loon et al., 1998), whilst others induce resistance to insect pests (Zehnder et al., 1999; Ramomoorthy et al., 2001). Many of the growth enhancing bacteria are referred to as PGPR (plant growth promoting rhizobacteria) (Buchenauer, 1998).
Resistance can be mediated through distinct signaling cascades in the plant – despite the biological end result being the same – depending on the type of signal or stimulus that is produced by the inducing bacteria. "Systemic acquired resistance", abbreviated SAR (Kuc, 1983, 1987), is induced in response to agents such as plant pathogenic microorganisms, where salicylic acid is a central intermediate (Kessmann et al., 1994). Induced systemic resistance (ISR) is a term that has been employed to describe a pathway to resistance that is mediated through nonpathogenic microorganisms, and for which jasmonic acid is an intermediate (Van Loon et al., 1998). SAR is known to mediate resistance to insect pests as well as various plant pathogens (Inbar et al., 1998; Hammerschmidt & Kuc, 1995). Zehnder and coworkers (1997a & 1997b) treated seeds of cucumber and soil for sowing with PGPR and noted a significantly lower level of attack of leaf beetles (Acalymma vittatum and Diabrotica undecimpunctata) in the plants whose seeds had been treated with bacteria. The first trials were conducted in greenhouses for short periods of time. However, in field grown cucumber whose seeds had been treated, the effect was observable close to two months after field planting of seedlings. The resistance observed in these trials was tentatively designated ISR (Zehnder et al., 1999).

In our collection of microorganisms we have nonpathogenic, environmental isolates of bacteria that have been screened and selected for their biocontrol properties to Fusarium diseases of wheat (Johansson et al., 2003) and barley leaf spot (Hökeberg et al., 1997). We describe below how some of these isolates also have the ability to induce resistance in barley to feeding of leafhoppers (Psammotettix alienus) (Dahlbom) and to incidence of leaf rust (Puccinia hordei) and powdery mildew (Blumeria graminis f. sp. hordei). Powdery mildew is considered to be one of the most important fungal diseases of barley (Wiik et al., 1995).

Materials and methods

**Bacterial strains, barley cultivars, fungal isolates, insects, growth conditions and media**

The following bacterial isolates were used in this study: Pseudomonas chlororaphis strain MA342 (Hökeberg et al. 1997); several pink pigmented facultative methylotrophic bacterial isolates from Morocco: MA2, MA3, MA4, MA5, MA6, MA7, MA8, MA9, MA10, MA11, MA14, MA15, MA16, MA18, MA19, MA22 and MA23 (Saad Omer, 2004); and isolates MF30, MF174, MF181, MF200, MF231, MF299, MF304, MF400, MF417, MF434 and MF588 (Johansson & Wright 2003) isolated in Sweden and Switzerland. The bacteria were grown routinely on Tryptic Soy Agar (TSA) (Difco, Becton Dickinson, Le Pont de Claix, France) at 22°C. For seed application, the MF isolates and MA 342 were grown in Tryptic Soy broth (Difco) for 48 hours at 19 to 22°C. Prior to seed application, the Moroccan (MA) isolates were cultivated on TSA plates at 27°C for five days. The bacterial growth on each plate was resuspended in 10 ml tap water. For powdery mildew trials, the two near isogenic lines of the cultivar 'Pallas' P01 and P02 (Kølster et al., 1986) were used. They have race specific resistance to the powdery mildew races A6 and C15, respectively. These races were used in the experiments. The barley cultivar 'Ingrid' is susceptible to leaf rust and was used to test induction of resistance to leaf rust. The rust isolate used was ND97-21. It originates from Brian Steffenson, Univ. of Minnesota, St. Paul and was kindly provided by Dr. Anders Falk, Dept. of Plant Biology and Forest Genetics, SLU, Uppsala, Sweden. Leafhoppers of the species P. alienus were collected in the field (Azrang, 1976). They were maintained at 25°C in a ventilated rearing cage in the greenhouse. For leafhopper experiments, three to four leafhoppers were removed from the rearing cage with a suction apparatus and placed in an experimental cage consisting of a stocking that had been placed over bent wires over a pot. Leafhopper experiments were carried out at 25°C. The leafhoppers were placed on plants of 1 decimeter length (2nd leaf stage) and their feeding preference was recorded four times a day for a period of 10 to 14 days.
**Bacterial application**

A suspension containing approximately $10^8$-$10^{10}$ cfu/ml, were applied to the seeds, in accordance with the established procedure (Hökeberg et al., 1997). Treated and untreated seeds were sown in separate pots. The pots were placed in the greenhouse at 23 to 25°C.

**Powdery mildew and leaf rust trials**

Forty eight hour broth cultures of the 28 isolates listed above (MA and MF series) were applied to seeds (150 ml culture broth per 150 seeds). These isolates were screened for the ability to induce resistance to powdery mildew in P01 and P02. When the plants had reached a height of 1 decimeter, they were challenged with powdery mildew. Conidia were shaken over the first leaf of emerging barley plants. The number of spores per leaf area was counted according to the method described by Thordal Christensen & Smedegaard Petersen (1988). Methods for inoculation of powdery mildew and scoring of symptoms are described in Cho & Smedegaard Petersen (1986) and in Haugaard et al. (2001). The inoculated plants were placed at 19°C in the greenhouse. The number of powdery mildew colonies per 4 centimeters of leaf length was counted for six pots and five leaves per pot for every treatment. For leaf rust experiments, the leaves were spray inoculated with a method that we developed (data not shown) when the leaves had reached a height of one decimeter. The plants were covered with plastic overnight. When initial rust pustules appeared, leaves were removed and weighed, and the number of spores per mg leaf was estimated. Spores were washed from the leaves and suspended in tap water containing a droplet of soap and counted in the microscope.

**Statistical analysis**

The data were analyzed with Anova and differences between means of the treatments were tested with Duncan’s multiple range test at $P<0.05$.

**Presence of bacteria in aerial plant parts**

The presence of bacteria in aerial plant parts was checked. Seeds treated with isolates MF231, MF299, MF304 and MF417 were sown in separate pots, five pots per treatment. Untreated seeds were sown as a control. The plants were grown at 20°C. When the plants had reached a height of 1 decimeter, a leaf disk from one leaf per pot was taken at a random location, surface sterilized in 70% ethanol and crushed with mortar and pestle in 1 ml MgCl$_2$. The suspension was serially diluted and plated on TSA with or without supplement of cycloheximide.

**Results and discussion**

Plants originating from seeds treated with some of the isolates were less attractive as a source of food for *P. alienus*. The mean visitation frequency was significantly greater on untreated plants than on plants whose seeds were treated with the following isolates: MA 342, Ki95, MV94 and MF29. These isolates were known from previous work to provide protection against fungal seed borne diseases of cereals (Johansson et al., 2003; Borowicz, 1998). The Moroccan isolates (MA isolates) and MF30, MF174, MF181, MF200, MF231, MF299, MF304, MF400, MF417, MF434 and MF588 were not tested for induction of insect resistance.

Among the MA isolates, MA14 consistently provided protection from powdery mildew (in two tests). Seeds treated with MA14 produced plants that were significantly less attacked by the fungus than those of the other treatments and that of the untreated control. There was less incidence of powdery mildew on plants that had originated from seeds treated with MF299, MF231, MF304 and MF417. The reduction of powdery mildew incidence ranged from 29 to 44% for MF231, from 21 to 63% for MF304 and from 31 to 38% for MF417. Similar results were obtained on several occasions, and these four isolates were selected for
subsequent field trials. Isolate MF304 was the most effective of the MF isolates in providing protection against leaf rust.

We attempted to isolate bacteria from aerial plant parts to ascertain that the effect we saw was not due to a direct interaction of the isolates with the powdery mildew fungus, the rust fungus or the leafhoppers. Suspensions containing surface sterilized and crushed leaf disks did not contain any traces of the bacterial isolates that had been applied to the seeds. In another experiment, seeds were coated with a version of strain MA 342 that had been tagged with a double copy of the gene for green fluorescent protein (Tombolini et al., 1999). Nonsterile, crushed barley leaves were plated on kanamycin containing medium, and no bacteria were recovered then either (data not shown). These results indicate that the bacterial isolates do not travel through the plant nor in any other way come in contact with the pathogens or insect pests used as challenge treatments. It is therefore likely that the bacterial isolates of this study are inducing a resistance response in the plants.

Several other factors, such as plant growth regulating substances, herbicides, mechanical damage, BTH (a synthetic form of salicylic acid), jasmonic acid and some insect pests (Kogan & Paxton, 1983; Fischer et al., 1990; Green & Ryan, 1972; Inbar et al., 1998; Thaler, 1999; Stout et al., 1998) have also been reported to induce resistance. Induction of resistance to powdery mildew has been documented by several research groups, for instance by Smedegaard Petersen's group in the mid 1980's (Cho & Smedegaard Petersen, 1986; Thordal Christensen & Smedegaard Petersen, 1988). These researchers managed to induce a high level of resistance by first treating the barley leaves with (incompatible) virulent and avirulent forms of *B. graminis f. sp. hordei*. This pretreatment protected the leaves from the subsequent challenge with a virulent (compatible) race of the fungus. Their results were impressive. However, it is difficult to imagine how this method could be developed for use in practical agriculture. In the case of the microorganisms described in this work, it is conceivable that they could be developed for use in commercial agriculture, since seed application methods for large scale production have been developed. In the future, when field efficacy of isolates has been established, we foresee the development of this work for use in practical agriculture.

Acknowledgements

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References


Integrated control of diseases
Integration of *Trichoderma* and soil solarization for disease management

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**Abstract:** Soil solarization and *Trichoderma* application are used to suppress pathogens and have a positive effect on plant growth and yield. We investigated the effects of solarization and *T. harzianum* T39 soil treatments, alone or combined, on yield and on disease suppression, especially when a plant growth medium was reused. *Trichoderma* was applied after solarization. Reusing the substrate growth medium ('old medium') reduced yield, but the above treatments counteracted the negative effects of the 'old medium'. *Trichoderma* application to soil, solarization or combination of both treatments were frequently as effective as using a new medium, thus enabling reusing the growth media. Strawberry, cucumber and beans grown on treated 'old' soil or growth substrate showed significant reduction in disease after leaf inoculation with *Botrytis cinerea* (grey mould) or with *Sphaerotheca fuliginea* (powdery mildew) on cucumber, hence indicating induced resistance. No significant changes in culturable microorganism populations, except for increased population levels of bacteria in rhizosphere of plants treated with the *Trichoderma*, were detected when using the method of dilution on selective agar media for microorganism enumeration. Attempts are being made to use a molecular technique based on 16S ribosomal DNA fingerprints. Denaturant gradient gel electrophoresis (DGGE) reveals different band patterns of rhizosphere populations of the different treatments.

**Keyword:** *Trichoderma*, soil solarization, disease management, induced resistance.

**Introduction**

Plants are frequently exposed to a variety of pathogens, in the same soil, which all have to be controlled. This can be achieved by using a broad spectrum pesticide, which is frequently highly toxic, e.g. methyl bromide. Alternatively, integration of methods for disease management, if appropriately applied, results in improved and a wider spectrum of disease control, while reducing chemical use.

Soil solarization is a method for soil disinfestation, which is based on solar heating of the soil. It is effective in controlling a variety of soilborne pathogens, e.g. *Fusarium, Verticillium, Rhizoctonia* and others (Katan & DeVay, 1991). Mechanisms of control are both physical and biological. Solarization also frequently brings to plant growth enhancement and improved yield, even in the absence of major pathogens (Gruenzweig et al., 1993). *Trichoderma* spp. are soil fungi antagonistic to many pathogens. *Trichoderma hamatum* induced suppressiveness of the soil to *R. solani* (Chet & Baker 1980, 1981). *Trichoderma harzianum* decreased damage of *R. solani* in strawberry daughter plants up to 46% and early yield increased by 21-37% (Elad et al., 1981). Combination of both soil solarization and *T. harzianum* resulted in improved control of various pathogens such as *R. solani, Fusarium oxysporum* f. sp. *radicis-lycopersici, Sclerotium cepivorum* and *Fusarium* in carnations, as
well as in yield increase (Chet et al., 1982; Elena & Tjamos, 1997; Katan, 1996; Ordentlich & Chet, 1989; Pereira et al., 1996; Sivan & Chet, 1993).

Induced resistance occurs when defense mechanisms of a plant are activated after being exposed to a biotic or abiotic inducing agent. Systemic acquired resistance occurs when an inducing agent is applied at a certain location and resistance is expressed in the whole plant or in a distant location (Kloepper et al., 1992, Kuc 1987). Isolate T39 of *T. harzianum* had an induced resistance effect similar to that obtained by *Pseudomonas aeruginosa* on bean, challenged by *B. cinerea* (De Meyer et al., 1998). T39 applied to the roots controlled powdery mildew on cucumber (Elad et al., 1998). The objective of this work was to study the effect of soil solarization and *Trichoderma* application to the growth medium or soil, on plant growth, induced resistance and microbial populations.

**Materials and methods**

**Experimental growing conditions**

Large-scale experiments of strawberry were conducted in two Israeli locations: Besor and Tzafit, in which the plants were grown for 7-8 months. Strawberry was grown in a commercial greenhouse on hanged polystyrene containers of 9 l volume each, on a growth substrate consisting of coconut residues, plus 15% polystyrene. The growth substrate was either freshly obtained (new) substrate or a substrate in which strawberries were grown in the previous season (old). Three treatments were carried out with the old substrate, in addition to untreated: 1. Solarization by mulching with transparent polyethylene for 40 days. 2. Application of *T. harzianum* T39 by pouring TRICHODEX (20% *T. harzianum* T39 Makhteshim, Beer-Sheva) suspension (3g/L substrate) to the substrate. 3. Combination of solarization and *T. harzianum* T39. Smaller scale greenhouse experiments for induced resistance with cucumber, common bean and strawberries were conducted as described below.

**Microbial populations**

Enumeration of total number of fungi, bacteria and fluorescent pseudomonads was carried out with the dilution method using specific selective agar media (Gamliel and Katan, 1991). Populations of *Trichoderma* spp. were enumerated using a selective agar medium containing 24 g/l potato dextrose agar amended with 0.25g/l chloramphenicol and 0.05g/l rose bengal.

**Denaturing gradient gel electrophoresis (DGGE)**

DNA extractions from rhizosphere were PCR amplified with general primers of 16S- rDNA of microbes and specific primers for 16S-rDNA for *Pseudomonas*, then loaded and run on the DGGE according to Muyzer et al. (1993).

**Induced resistance experiments**

Cucumber and common bean seeds were sown in nonsolarized, solarized, soil or coconut residue either with or without *T. harzianum* T39 treatment. When the plants reached the age of first leaf (after about two weeks), detached leaves were inoculated each with 1 mm disc of young culture of *Botrytis cinerea* grown on ¼ strength PDA. Strawberry leaves were inoculated when leaves were fully grown. The inoculated leaves were kept in sealed boxes containing wetted sheets, at room temperature, for three-four days. Diameter of the expanding diseased tissue was measured and the rot area was measured. Inoculation with the cucumber powdery mildew pathogen *Sphaeroteca fuliginea* was conducted on 4 weeks old plants and was carried out by blowing conidia off infected leaves. The inoculated plants were maintained in a greenhouse at temperatures of 22-26°C. Disease symptoms appeared after 7-10 days and were evaluated as percentage of leaf coverage.
**Statistical analyses**

Results of microbial enumerations are means of three replicates; each represents a composite sample from five plants. Disease data for *B. cinerea* are means of at least four replicates, each is the mean of two inoculations on one leaf for cucumber and common bean and six inoculations on one leaf for strawberry. Disease data for *S. fuliginea* are means of at least five leaves of different plants. All tests were repeated once or more. Analyses test were conducted with the JMPin 5.0.1 software according to the Tukey Cramer Test ($P<0.05$).

**Results**

**Effect on strawberry yield**

Yield of strawberry grown on old (previously used) coconut residues was significantly lower than the yield of strawberry grown on a new medium (Figure 1). The combined treatment of solarization and *Trichoderma*, compensated for the loss of yield obtained on old medium, thus the yield did not differ significantly from a new medium. A similar trend was obtained in additional experiments. In some cases, solarization or *Trichoderma* alone significantly increased the yield of strawberry grown on old medium.

![Figure 1. Effect of solarization (sol) and *Trichoderma harzianum* T39 (Trich) treatments, alone or combined, on strawberry yield grown on old or new coconut residues. Bars with different letters are significantly different from each other ($P<0.05$). Old = Previously used substrate; New = Fresh substrate.](image)

**Microbial populations**

Generally, the microbial populations examined (total number of fungi, bacteria, *T. harzianum* and fluorescent pseudomonads) in the rhizosphere of plants originating from various treatments did not differ significantly from each other. The main exception was that *Trichoderma* treatment increased the population of the total bacteria in the rhizosphere; $10^8$ cfu/g and $10^9$-$10^{11}$ cfu/g in untreated and *Trichoderma* treated media, respectively. *Trichoderma* populations in the *Trichoderma* treated substrates were also higher than in the untreated ones by 2-3 orders of magnitude.

We used PCR-DGGE for assessing microbial changes in the rhizosphere. When using universal primers for bacteria, different band patterns were obtained in rhizosphere from plants grown on untreated soil compared to plants grown on solarized treated soil (results not shown). Specific primers for pseudomonads also showed changes in band patterns (results not shown). Efforts are now being made to sequence the differential bands and to determine the specific microbes involved.

**Induced resistance**

*T. harzianum* T39 or solarization significantly reduced disease severity of *B. cinerea* in detached leaves of cucumber, strawberry and common bean (Figure 2). The combined
treatment reduced disease even more, namely by 60%, 95% and 90% for bean, strawberry and cucumber, respectively. Results obtained with whole plants (results not shown) were similar to results from detached leaves. Similar results were obtained when the plants were grown on either a sandy soil or coconut residues medium. The above treatments also affected powdery mildew disease. After seven days from inoculation, percentage leaf coverage by *S. fuliginea* on cucumber plants grown in treated soil was significantly lower (Figure 3). After 10 days the combined treatment significantly reduced percentage leaf coverage by about 85%, compared to untreated. Induced resistance was less pronounced at higher disease incidence.

Discussion

Treatment with solarization or *T. harzianum* resulted in microbial changes in the plant rhizosphere, induced resistance to *B. cinerea* and *S. fuliginea* and in higher strawberry yield. The integrated treatment was even more effective. Combining methods of control may provide a better and wider spectrum of control and even for a longer term effect (Katan, 1996). Integrated control may result in either an additive or a synergistic effect. Soil solarization alone is known to cause changes in the soil microbial populations (Gamliel and Katan, 1991) and to have beneficial effects such as improved plant growth beyond pathogen control (Gruenzweig et al., 1993). We found that *T. harzianum* increased the bacterial population. It is possible that the improved plant growth observed in the *Trichoderma* treatment (Figure 1) is due to a direct effect of *Trichoderma* or due to an increase in populations of beneficial microbes. We attempt to follow that using the molecular method of
DGGE. It is also possible that the beneficial effect of combination of solarization and Trichoderma is due to a shift in the microbial population to a beneficial one. Improvement in disease control, yield increase, or both, have been demonstrated by combining biological control by Trichoderma with solarization in other studies (Chet et al., 1982; Elena & Tjamos, 1997; Katan, 1996; Ordentlich & Chet, 1989; Pereira et al., 1996; Sivan & Chet, 1993). Weakening propagules of pathogens by means of solarization may render the pathogen more vulnerable to the biocontrol agent, which in particular may add to the control effect by Trichoderma (Katan, 1996).

In our experimental system on induced resistance, the treatments were applied to the soil, or to the coconut residues growth substrate, and the plants were challenged above ground. Hence, the treated organ remained spatially separated from the pathogen on the leaves. In the case of plants with B. cinerea, the leaves were also inoculated after being detached from the plants, thus were further separated. It seems that these separated leaves withheld the inducing effect of the treatment, indicating that some signals or physiological changes were received or transferred to the leaves.

Soil solarization aims to control soil borne pathogens upon heating the soil. Nevertheless, there are surprising results of reduction of foliar diseases by solarization. This phenomenon was attributed to the control of the primary inoculum in the soil. However, induced resistance, triggered by the solarized soil, should also be considered, as also shown in studies at Alabama (Stevens et al., 2002). Soil solarization and Trichoderma application to the soil may affect the indigenous microflora, and these in turn may affect plant growth. Integration of soil solarization and Trichoderma amendment to the growth substrate or soil enable reusing growth medium and achieving good yield. This became very important recently, since methyl bromide, which is the major soil fumigant, will be phased out by 2005. Combining methods of control with pesticides at reduced dosage should also be considered.

References

Integrated control of *Allium* white rot using biological control agents, composted onion waste and tebuconazole treated seed

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**Abstract:** Two isolates of *Trichoderma viride* were found to be effective fungal biological control agents (BCAs) of *S. cepivorum*. In laboratory tests where BCAs were added as wheat bran cultures to *S. cepivorum* sclerotia in soil at different temperatures, degradation of sclerotia occurred at 5-25°C after 8 weeks with 74-86% sclerotia degraded at 20-25°C. In a glasshouse bioassay where BCAs were added to soil infested with sclerotia and onion seed planted, *Allium* white rot (AWR) was reduced by at least 40% for five commercial onion varieties. Similar standard glasshouse bioassays were also used to test the efficacy of BCAs when combined with either composted onion waste or tebuconazole seed treatments. Control of AWR was better in the combination treatments than with any treatment alone and over 90% control was achieved. Combinations of BCAs, composted onion waste and tebuconazole seed treatments therefore have great potential as an integrated system for AWR control.

**Keywords:** *Allium* white rot, *Sclerotium cepivorum*, *Trichoderma*, biological control, tebuconazole, onion compost

**Introduction**

*Allium* white rot (AWR) caused by the soil-borne fungus, *Sclerotium cepivorum*, is a major problem for the onion-growing industry world-wide. In the UK, large areas of onion-growing soils have been lost due to AWR infestation and chemical control is limited to tebuconazole treatments (Melero-Vara et al., 2000; Dennis, 2001). Recently however, Coventry et al., (2002) have demonstrated the potential of composted onion waste to control AWR in glasshouse trials. Over 30,000 tonnes of onion waste are produced in the UK annually and disposal is becoming a problem as landfill sites become more expensive and with EU legislation restricting disposal options. This paper describes the effect of BCAs on AWR, both alone and in combination with tebuconazole seed treatments and composted onion waste. The aim of this work is therefore to develop an integrated strategy for AWR control using alternative and sustainable methods.

**Materials and methods**

**Sclerotial degradation test**
*T. viride* isolates S17A and L4 were used as BCAs of AWR. They were applied (0.2 g) as 3-day-old wheat bran cultures (incubated at 20°C) to *S. cepivorum* sclerotia (100) mixed with soil (10 g) in mesh bags as described by Clarkson et al. (2002). The bags were buried in soil contained in plastic boxes (600 ml volume) and the soil water content maintained at 75% field capacity. Boxes were incubated at 5, 10, 15, 20 and 25°C for 8 weeks. After this period, sclerotia were recovered and assessed for degradation (soft or collapsed).
Glasshouse tests – BCAs alone
*T. viride* isolates S17A and L4 were applied as 3-day-old wheat bran cultures (1 g per 100 g soil) to soil infested with conditioned sclerotia of *S. cepivorum* (3 sclerotia per g soil) as described by Clarkson et al. (2002). The soil was thoroughly mixed before it was distributed into pots and sown with 5 different varieties of commercially available onion seed (one seed per pot; White Lisbon, Hysam, Renate, Red Baron and Summit). The control treatment consisted of soil and sclerotia only (no *Trichoderma*). Pots were placed in a glasshouse (min. temperature 15°C) and onions were assessed for AWR symptoms weekly until no further plants became infected.

Glasshouse tests – BCAs combined with tebuconazole seed treatment or onion compost
To test combinations of BCAs and tebuconazole treated onion seed, the *T. viride* isolates were added as wheat bran cultures to soil infested with conditioned *S. cepivorum* sclerotia as described before. Treated or untreated onion seed (White Lisbon) was then sown in the pots. Control treatments were sclerotia with treated / untreated onion seed only and sclerotia with BCA only. Onions were assessed for AWR symptoms weekly until no further plants became infected.

To test combinations of BCAs and composted onion waste, soil was infested with conditioned sclerotia and then amended with onion compost (50% v/v). The compost was produced by incubating onion waste in flasks at 50°C for 7 days as described by Coventry et al. (2002). The onion compost and infested soil was mixed in bags and left in the glasshouse at approx. 15°C for 12 weeks. After this time, *T. viride* isolates were added as wheat bran cultures as before and the soil/sclerotia/BCA/onion compost was thoroughly mixed and dispensed into pots which into which onion seedlings were transplanted (White Lisbon). Control treatments were sclerotia only, onion compost only and sclerotia with BCA only. Onions were assessed for AWR symptoms weekly until no further plants became infected.

Results

Sclerotial degradation test
Degradation of *S. cepivorum* sclerotia by *T. viride* L4 and S17A increased with temperature and reached a maximum of 74-86% at 20-25°C (Table 1). There was little degradation of control sclerotia (no BCAs applied) at 5-15°C and 15-17% degradation at 20-25°C.

Table 1. Percentage degradation of *S. cepivorum* sclerotia at different temperatures by *T. viride* L4 and S17A after 8 weeks in soil

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5°</td>
</tr>
<tr>
<td>No BCA</td>
<td>1.6</td>
</tr>
<tr>
<td><em>T. viride</em> L4</td>
<td>10.7</td>
</tr>
<tr>
<td><em>T. viride</em> S17A</td>
<td>13.6</td>
</tr>
</tbody>
</table>

Glasshouse tests – BCAs alone
*T. viride* isolates L4 and S17A reduced AWR for all the commercial onion varieties tested and percentage reduction in disease ranged from 43-75% after 11 weeks (Figure 1).
Figure 1. Effect of *T. viride* L4 and S17A on AWR for 5 commercial onion varieties

**Glasshouse tests – BCAs combined with tebuconazole seed treatment or onion compost**

*T. viride* isolates L4 and S17A reduced AWR when combined with tebuconazole-treated or untreated onion seed (Figure 2). Compared to the untreated control, percentage disease reduction was 68% and 69% respectively for L4 and S17A with untreated seed after 22 weeks and was 96% and 92% when combined with the treated seed. The tebuconazole treated seed without any BCA reduced AWR by 51%.

When *T. viride* isolates L4 and S17A were combined with composted onion waste, less than 1% of onion plants became diseased with AWR after 18 weeks (Figure 3). This represented a disease reduction of almost 100% compared to the untreated control. Composted onion waste alone reduced AWR by 96% and in the same experiment, *T. viride* L4 and S17A without onion compost reduced AWR by 81%.

Figure 2. Effect of *T. viride* L4 and S17A on AWR with and without tebuconazole treated seed

Figure 3. Effect of *T. viride* L4 and S17A on AWR with and without composted onion waste
Discussion

*T. viride* isolates L4 and S17A degraded *S. cepivorum* sclerotia and reduced AWR disease. Most degradation of sclerotia occurred at 20-25°C but some also occurred at temperatures of 5-10°C and this may have increased if the incubation period had been extended beyond 8 weeks. This does however suggest that the *Trichoderma* isolates have some activity at low temperature which may be beneficial if they were to be applied in the field before spring-sown onion crops.

AWR disease was controlled by BCAs in the absence of any other treatments in all experiments and control ranged from 43-81%. The level of AWR suppression was therefore variable. This may be due to different batches of sclerotia used or the prevailing conditions in the glasshouse at the time. However, when the BCAs were combined with either the tebuconazole treated seed or the composted onion waste, AWR control was enhanced and was greater than with any one treatment alone. An integrated system using combinations of these control measures therefore has great potential for control of AWR. Further work will now test the efficacy of an integrated control programme in the field.

Acknowledgements

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References


Latest results on the biocontrol of fire blight in Germany

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Abstract: As alternatives to the antibiotic streptomycin against fire blight bacterial antagonists and a natural product were tested on their efficacy in greenhouse and field experiments. The antagonistic strain of _Rhanella aquatilis_ - Ra39 in combination with aromatic compounds could increase the efficacy up to 68%. The etheric oil compound BioZell-2000B was able to reduce blossom infection up to 52% and is proposed as a bioproduct for fire blight in ecological farming.

Key words: fire blight, biocontrol, bacterial antagonists, natural product

Introduction

Research on alternatives to the antibiotic streptomycin for the control of fire blight in pome fruits has become of main interest in German and European fruit growing, as permission for the use of antibiotics in EEC-countries can be withdrawn in future time. Thus the development of biologicals on the basis of antagonism and natural products against the pathogen have been undertaken in Germany especially by the Federal Research Centre (BBA) in cooperation with plant protection service. Latest results with antagonistic bacteria and a natural product based on an etheric oil of thyme, BioZell-2000B, which was developed together with Turkish colleagues (Yegen et al., 2002) will be presented.

Results and discussion

Studies on bacterial antagonists

First screening of several potential antagonists of more than 120 isolates, showed inhibitory effects mainly of the species _Pseudomonas fluorescens, Pantoea agglomerans_ and _Bacillus subtilis_ (Zeller & Wolf, 1996), later on also from the epiphytic bacterium _Rahnella aquatilis_ (Laux et al., 2002). Moreover in field experiments efficacies up to 61% could be observed (Table 1) but without a similar control effect to streptomycin. In order to increase the efficacy of strain Ra39, the antagonist was combined with aromatic compounds, as it was lower sensitive to benzoate as the fire blight pathogen in vitro and was able to use this bactericidal substances as nutritive source (Figure 1) In field experiments the combination of Ra39 and Na-Benzoate was nearly comparable in the efficacy to streptomycin, 68 to 77%. Also another combination with the growth regulator REGALIS (APOGEE) showed high control effect of 68% (Figure 2).

Studies on natural products

As another biological alternative the plant strengthener BioZell-2000B, a compound out of 70% Thyme oil from _Thymbra spicata_, which has been registered in Turkey as a plant protection substance (Yegen et al., 2002), was tested on its efficacy against fire blight under greenhouse and field conditions.
Table 1. Efficacy of bacterial antagonists against blossom infection, 1998-2001

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Origin</th>
<th>Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> BsBD 170 (BIOPRO)</td>
<td>Soil</td>
<td>30-60</td>
</tr>
<tr>
<td><em>Rahnella aquatilis</em> Ra39</td>
<td>Apple blossom (Steinbrenner, 1991)</td>
<td>39-53</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td>Shoot tissue</td>
<td>45-58</td>
</tr>
<tr>
<td><em>Pantoea agglomerans</em> Pa21889</td>
<td>Apple Blossom (Steinbrenner, 1991)</td>
<td>50-61</td>
</tr>
<tr>
<td>Control</td>
<td><em>Streptomyces griseus</em></td>
<td>68-80</td>
</tr>
</tbody>
</table>

Figure 1. Effect of Na-benzoate on the growth of *Ra39* and *E. amylovora* strain-Ea 7/74 *in vitro*

![Graph showing the effect of Na-benzoate on the growth of Ra39 and E. amylovora strain-Ea 7/74.](image)

Figure 2. Fire blight control with Ra 39 in different combination on Golden Delicious after natural infection, 2003

![Bar chart showing infected blossoms (%) after different treatments.](image)

Firstly, under *in vitro* conditions in a concentration of 0.05% no direct effect of BioZell-2000B on the pathogen could be observed indicating the plant strengthener effect of the compound. In greenhouse symptom development on leaves of the highly susceptible apple rootstock was markedly reduced after two days induction time with BioZell-2000B (Figure
3). Under field conditions on apple variety Boskoop significant reduction of blossom infection of 52% was found (Table 2). In a further control experiment on the highly susceptible Cotoneaster variety *C. salicifolius* the control effect could be confirmed with 85%.

Table 2. Fire Blight control with BioZell-2000B on apple variety Boskoop after natural blossom infection, 2001

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of Blossom clusters</th>
<th>Infection (%)</th>
<th>Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Infected</td>
<td></td>
</tr>
<tr>
<td>Inf.control</td>
<td>720</td>
<td>70</td>
<td>9.7 a</td>
</tr>
<tr>
<td>BioZell-2000B</td>
<td>680</td>
<td>30</td>
<td>4.7 b</td>
</tr>
</tbody>
</table>

Figure 3. Disease index of fire blight on shoots of M26 rootstock after treatment with Bio-Zell-2000B

References


Effect of fungicides and herbicides on in vitro sensitivity of *Clonostachys rosea* and different strains of *Trichoderma*

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Abstract: Five fungicides and four herbicides were tested to evaluate their in vitro effects on colony radial growth (MG) and conidial germination (CG) of *Clonostachys rosea* 47 (CR47), *Trichoderma atroviride* 59 (TA59), *T. atroviride* 312 (TA312), *T. harzianum* 24 (TH24), *T. longibrachiatum* 9 (TL9), *T. longibrachiatum* 144 (TL144) and *T. viride* 15 (TV15). All antagonists were not sensitive to carboxin for CG and sensitive to prochloraz for CG and MG. Most of the antagonists were stimulated by the herbicides applied at field dose for MG. The effects of the fungicides on the antagonists mycelia were confirmed by studies under light (LM) and scanning electron microscopes (SEM).

Key words: *Clonostachys rosea*, *Trichoderma* spp., fungicides, herbicides, sensitivity

Introduction

Fungicides and herbicides widely differ in their chemical structure and functional groups, and have different effects on various fungi. Hence, detailed information on the susceptibility of fungi, used as biocontrol agents (BCA), before their application to the crop, is needed. *Trichoderma* spp. is a BCA tolerant to many fungicides (Elad et al., 1981; Davet et al., 1981; Figueras Roca et al., 1996; Tewari et al., 2003), but it is not well known its reaction to the herbicides (Ruppel et al., 1988; Jayaraj & Radhakrishnan, 2000; Jaworska & Dluzniewska, 2002). *Clonostachys rosea* (*Gliocladium roseum*) resulted to be sensitive in vitro only to prochloraz (Nan et al., 1991).

Our previous research, on biological wheat seed treatments with *C. rosea* (*G. roseum*) and *Trichoderma* spp. against *Fusarium* spp., responsible for wheat foot and root rot, showed that these fungi could partially control the disease in greenhouse and in the field, by 56-76% (Roberti et al., 1996; Roberti et al., 1997; Roberti et al., 2000). The aim of this research was to test in vitro the effects of some fungicides and herbicides, usually applied in Italian wheat cultivation, towards *C. rosea* and *Trichoderma* spp. in order to integrate them in a wheat crop management.

Material and methods

The antagonistic fungi, *Clonostachys rosea* 47 (CR47), *Trichoderma atroviride* 59 (TA59), *T. atroviride* 312 (TA312), *T. harzianum* 24 (TH24), *T. longibrachiatum* 9 (TL9), *T. longibrachiatum* 144 (TL144) and *T. viride* 15 (TV15), were isolated from wheat crown,
loamy soil, horticultural plant roots and seeds and their monospore colonies were maintained on PDA (4%, Difco). They were identified by CBS (Baarn, The Netherlands) and the code numbers refer to the position in the collection of the DiProVAl, University of Bologna, Italy. The effect on in vitro mycelial radial growth (MG) and conidial germination (CG) of the antagonists excreted by fungicides carboxin, guazatine, prochloraz, thiram and triticonazole and the herbicides chlorsulfuron, chlortoluron, flufenacet and pendimethalin were tested.

Five concentrations of fungicides, from 1/10 of the field dose followed by progressive 1/10 dilutions, and four concentrations of herbicides, the first corresponded to the field dose followed by progressive 1/10 dilutions, were applied. MG was assessed by inoculating each treated PDA plate, with agar plugs from the margin of 7-day-old colonies. Four replicates were made for each fungicide and herbicide concentration. The plates were incubated at 25 ± 1°C in the dark. Colony diameter was measured from time 0 to 96 h every 24 h. Conidia, from each 7-day-old antagonist colony, were added to 2% malt extract to make a suspension of 10^6 conidia/ml final concentration. The suspension was mixed with four different concentrations of each chemical and incubated at 25 ± 1°C in the dark for approximately 24 h. The percentage of germinated conidia was evaluated using a light microscope (LM). The sensitivity of each antagonist to the fungicide active ingredients was expressed as EC_{50} value (effective concentration of active ingredient required for 50% MG inhibition or CG), while the sensitivity to each herbicide, was expressed in terms of percentage of MG and CG of the antagonists. The effects of the fungicides and herbicides on the antagonists were studied at light (LM) and scanning electron microscopes (SEM) (Pisi & Filippini, 1994).

Results and discussion

Various degrees of sensitivity between antagonistic fungi and fungicides or herbicides were found. All antagonists showed no sensitivity to carboxin and triticonazole for CG, and moderate to high sensitivity to prochloraz for MG and CG (Table 1). TV15 and TH24 were the least sensitive for MG and CG except for some cases, TL144 was the most sensitive except for carboxin for CG and CR47, for either MG and CG, was sensitive only to prochloraz.

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Carboxin</th>
<th>Guazatine</th>
<th>Prochloraz</th>
<th>Thiram</th>
<th>Triticonazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR 47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA 59</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>TA 312</td>
<td>–</td>
<td>–</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>TH 24</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>TL 9</td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>TL 144</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>TV 15</td>
<td>–</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. Behaviour of mycelial radial growth (MG) and conidial germination (CG) of the antagonists, after 5 days of incubation with the selected fungicides

+++ highly sensitive, EC_{50} < 1·10^{-3} ; +++ very sensitive, EC_{50}: 1·10^{-2} ÷ 1·10^{-3} 
++ moderately sensitive, EC_{50}: 1·10^{-1} ÷ 1·10^{-2}; + low sensitive, EC_{50}: field dose ÷ 1·10^{-1} 
– no sensitive, EC_{50} not detectable.
All antagonists were almost insensitive to the four tested herbicides, applied at field dose, and their MG was differently stimulated, except for TL144 which was slightly inhibited in presence of chlortoluron (Figure 1A). The herbicides stimulated the CG of most antagonists, except for TA312; chlortoluron and flufenacet induced a slight reduction of TL9 and TL144 CG (Figure 1B).

![Figure 1. Effect of herbicides on mycelial radial growth (A) and conidial germination (B) of antagonists after 5 days of incubation](image)

The *in vitro* effects of the fungicides and herbicides towards the selected antagonists were confirmed by LM and SEM observations. Most of the antagonists sensitive to the fungicides showed, at LM, absence of conidia and hyphal disruptions and, at SEM, damaged hyphae with cell wall depression, degeneration and extrusion of cytoplasm content (Figure 2). No appreciable hyphal alteration by the herbicides was observed at LM and SEM.

![Figure 2. SEM micrographs: A, TA59 with 0.53 µg ·ml⁻¹ guazatine; B, TH24 with triticonazole 0.3 µg ml⁻¹; C: untreated control. Hyphal disruptions and extrusion of cytoplasm are visible in A and B. Bar = 10 µm.](image)

Based on our data, we need to continue this study since the sensitivity of the antagonists to the fungicides may be related to their inability to synthetize alternative molecules essential to their growth or to metabolise the fungicides in non toxic compounds. Further study of the interaction of the fungicides and herbicides with the antagonists in the integrated biocontrol of foot and root rot disease, caused by *F. culmorum*, in wheat crop is needed.
Acknowledgements

Research was supported by project COFIN “Capacità intrinseche ed indotte di difesa dai patogeni fungini nel seme e nella plantula” (MIUR) and project RFO “Microscopia elettronica a scansione: sue applicazioni in Patologia vegetale” (Alma Mater Studiorum - Università di Bologna).

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Strategies to provide integrated biological control of late blight of potato to replace copper for sustainable organic agriculture production

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Late blight (caused by Phytophthora infestans) is one of the most important diseases affecting organic and conventional potato production worldwide. Under suitable environmental conditions for the pathogen, the disease can spread very rapidly and can cause complete crop losses. Protective copper fungicides, which are currently used to control the disease in most organic production systems, are estimated to extend the length of the growing season by between 10 to 30 days. However, the amounts of copper fungicides allowed to be used in organic farming in the EU have recently been drastically reduced, and a complete ban is foreseen for the future. Therefore, alternative control treatment strategies are required.

For the control of some other fungal pathogens alternative treatments have already been developed, including microbial antagonists and also plant extracts, which have an effect on the fungus by direct antifungal activity, by stimulation of competitive microorganisms and/or by inducing plant resistance. In the framework of the EU-funded project “Development of a systems approach for the management of late blight in EU organic potato production – Blight-MOP” a screening with over 100 natural substances, including microorganisms and plant extracts, was carried out targeted to P. infestans. In addition, and in order to increase the control potential against P. infestans, compatible combinations of microorganisms and plant extracts were established. The tests were done on detached potato leaves and potted plants. In further studies in semi-field trials, optimal application intervals and persistence of the substances were tested. Application of the bacterial antagonist Xenorhabdus bovenii at two-day intervals resulted in good control of late blight. These methods, observations and results are reported here as attempts to further explore and develop integrated biological control methods for sustainable organic agriculture potato production.
Control of Phytophthora cryptogea with Trichoderma viride combined with furalaxyl and chitosan

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Abstract: Activity of Trichoderma viride isolates, obtained from diseased gerbera, as well as chitosan and furalaxyl toward Phytophthora cryptogea was studied in vitro and in greenhouse trials. In Petri dishes bioassays T. viride strongly inhibited the pathogen development. Mycoparasitism mediated by intimate hyphal interaction was observed. Chitosan only slightly suppressed the growth of the pathogen colonies. Amendment of peat with T. viride 10 days before infestation of substratum by P. cryptogea was the most effective. Combined application of furalaxyl with the mycoparasite was not as good as single application of the fungicide. Application of chitosan with T. viride 10 days before gerbera planting resulted in the strong decrease of P. cryptogea colony forming units number and increase of gerbera healthy stand to 60%.

Key words: Trichoderma, Phytophthora, population, chitosan, furalaxyl, gerbera, control

Introduction
Phytophthora foot rot of gerbera (Gerbera jamesonii), caused by P. cryptogea Pethybr. et Laff., is one of the most dangerous disease of that plant grown under covering. The pathogen is also known as the causal agent of root and stem rot of pot flowers and field grown ornamental nursery plants. Losses caused by P. cryptogea varied from a low percentage sometimes even to 60%. Chemical compounds are mostly used for the disease control. Previous study of Orlikowski (1995) showed, that Trichoderma spp. and Gliocladium roseum may suppressed the development of P. cryptogea. In this study activity of Trichoderma viride isolates, obtained from diseased gerbera as well as furalaxyl and chitosan toward P. cryptogea and control of Phytophthora foot rot of gerbera were studied in vitro and in greenhouse trials.

Materials and methods
Isolates of T. viride and P. cryptogea obtained from gerbera with foot rot symptoms were used in all trials. Stock cultures were maintained on PDA at 24°C. In an in vitro bioassays 3 mm diam plugs of both isolates, taken from the edge of 7-day-old cultures, were put on 90 mm Petri dishes 2 cm apart and incubated 6 days at 24°C. Inhibition zone and overgrown of P. cryptogea colonies by T. viride was observed during 8 days. Additionally, penetration of P. cryptogea hyphae by the mycoparasite was observed under microscope. In greenhouse trials peat with pH 5.6 was infested with P. cryptogea (initial population density = 250 cfu/g of air dry substratum) was mixed with T. viride (1.5 x 10⁶ conidia/g and 0.3 g of inoculum/g of air dry substratum). The mycoparasite was applied together with the pathogen, 10 days before gerbera planting. In trials with chitosan (2% of the commercial product Biochikol 020 PC) the compound at conc. 1000 µg/cm³ was applied together with T. viride 10 days before gerbera planting or immediately. Additionally, the compound was applied singly as substratum drench immediately after gerbera planting. Furalaxyl (a.i. of Fongarid 25 WP) at conc. 125 µg/cm³
was applied singly as peat drench immediately after gerbera planting or together with *T. viride* at concentration of 125 µg/cm³. One dm³ pots filled with infested substratum with gerbera were incubated on greenhouse bench at temperature varying from 17º to 27ºC. Influence of the mycoparasite and two tested compounds on population dynamic of *P. cryptogea* (Orlikowski, 1995, 1999) and development of *Phytophthora* foot rot of gerbera were studied. Experimental design was completely randomised with four replications and four Petri dishes and 5 gerbera plants in each rep. Trials were repeated 2 – 3 times.

**Results**

*Trichoderma viride* inhibited growth and overgrown colonies of *P. cryptogea* within 6-day-incubation. Mycoparasitism mediated by intimate hyphal interaction was observed. Application of *T. viride* alone, together with furalaxyl or chitosan and fungicide and biocide as single treatment 10 days before gerbera planting resulted in the significant decrease of *P. cryptogea* population density (Table 1). During the first 4 - 6 weeks of gerbera growth number of colony forming units of *P. cryptogea* in peat infested only with the pathogen (control) increased about three times whereas in combination with *T. viride* about two times. When the macoparasite was applied together with chitosan, the pathogen population density increased within the first two weeks about 75%. During the next four weeks of gerbera growth number of *P. cryptogea* cfu oscillated on the level of initial population density (Table 1). Mixture of *T. viride* with furalaxyl resulted in the decrease of the pathogen density below the initial level (Table 1). Application of chitosan as peat drench was less effective than its mixture with the mycoparasite in the suppression of the pathogen development, especially after four and six weeks of gerbera growth. Furalaxyl applied as substratum drench immediately after gerbera planting was the most effective in the inhibition of *P. cryptogea* development (Table 1).

Table 1. Influence of *Trichoderma viride* chitosan, furalaxyl and their mixtures, applied ten days before gerbera planting, on population dynamic of *Phytophthora cryptogea*; number of colony forming units (cfu)/g of air dry peat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weeks after gerbera planting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>378 cd</td>
</tr>
<tr>
<td><em>Trichoderma viride</em></td>
<td>293 c</td>
</tr>
<tr>
<td><em>Trichoderma viride</em> + chitosan 1000 µg/cm³</td>
<td>346 c</td>
</tr>
<tr>
<td><em>Trichoderma viride</em> + furalaxyl 125 µg/cm³</td>
<td>186 b</td>
</tr>
<tr>
<td>Chitosan 1000 µg/cm³</td>
<td>325 c</td>
</tr>
<tr>
<td>Furalaxyl 125 µg/cm³</td>
<td>45 a</td>
</tr>
</tbody>
</table>

Initial population density = 250 cfu /g of peat.
Means followed by the same letter do not differ at P<0.05 (Duncan’s multiple range test).

Analysis of the relationship between control agents application manner and healthiness of gerbera showed that furalaxyl applied alone or in mixture with T. viride were the most effective in the control of *Phytophthora* foot rot (Table 2). The mycoparasite applied alone significantly decreased the development of the disease but this effect was not satisfactory. Mixture of *T. viride* with chitosan was significantly more effective than peat application of the biocide alone (Table 2).
Table 2. Influence of *Trichoderma viride* chitosan, furalaxyl and their mixtures, applied ten days before planting, on healthiness of gerbera

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of diseased plants (n=10) after weeks of planting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>2.8 d</td>
</tr>
<tr>
<td><em>Trichoderma viride</em></td>
<td>1.5 bc</td>
</tr>
<tr>
<td><em>Trichoderma viride</em> + chitosan 1000 µg/cm³</td>
<td>1.8 c</td>
</tr>
<tr>
<td><em>Trichoderma viride</em> + furalaxyl 125 µg/cm³</td>
<td>0 a</td>
</tr>
<tr>
<td>Chitosan 1000 µg/cm³</td>
<td>1.0 b</td>
</tr>
<tr>
<td>Furalaxyl 125 µg/cm³</td>
<td>0 a</td>
</tr>
</tbody>
</table>

Means followed by the same letter do not differ with 5% of significance (Duncan’s multiple range test).

**Conclusions**

*T. viride* mixed with infested peat ten days before gerbera planting significantly decreased the number of colony forming units of *P. cryptogea*. Better effect was obtained when a mixture of *T. viride* with chitosan was applied. Application of *T. viride* together with furalaxyl was less effective in the suppression of the pathogen development than treatment of peat only with the fungicide. Furalaxyl alone or in mixture with *T. viride* were more effective in the control of *Phytophthora* foot rot than other treatments. A mixture of *T. viride* with chitosan applied 10 days before gerbera planting protected about 60% of gerbera. Chitosan applied alone was less effective.

**References**


Screening and identification of potential biocontrol agents against grapevine downy mildew considering an integrated control strategy of the disease

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Abstract: Grapevine downy mildew causal agent, Plasmopara viticola, is one of the most important grapevine pathogens, which causes high losses if chemical treatments are not applied. Nowadays some biocontrol agents are successfully used against several diseases. Due to its epidemiological characteristic (long lasting oospores, production of high quantity of sporangia, short time required for infection and destructive effect if the infection will take place in particular plant stages) the use of a single BCA is not sufficient to reach a satisfying control of downy mildew on grape. The aim of the present work was to find and evaluate micro-organisms that can limit development of P. viticola, to be used with an integrated approach as an alternative to chemical fungicides. Some effective micro-organisms against different stages of the pathogen have been identified.

Keywords: organic viticulture, BCAs, Plasmopara viticola, grapevine

Introduction

The grapevine downy mildew agent, Plasmopara viticola (Berk et Curt) Berl et De Toni, is one of the most important grapevine pathogens, which cause high losses if chemical treatments are not applied. Chemical control of the disease began in the 19th century using copper Bordeaux mixture (Gindro et al., 2003). At present the disease is controlled by the application of specific fungicides according to the epidemiological stages of the parasite (Viret et al., 2000). Nowadays there is an increased interest in biological control agents (BCAs) against plant diseases, due to the higher consumer and farmer awareness of health and environment safety, to the increased request of alternative product for disease control in organic agriculture, and, not at least, to the appearance of resistance to the active compounds of several modern fungicides against oomycetes.

Biological control is achieved through the activity of an organism on the pathogen target, which may reduce its inoculum or activity, finally resulting in less disease and disease impact. P. viticola has several characteristics (long lasting oospores, high sporangia production, short time required for infection, and destructive effect if the infection takes place in particular stages of the plant). Intrinsically BCA do not achieve 100% elimination, therefore it is doubtful that sufficient reduction of the disease impact can be achieved with a BCA targeted on a single stage. Therefore we focused our research on two pathogen stages: over-wintering and asexual reproduction, trying to identify BCAs active against one or both stages with the finality of developing a BCA or BCA-mixture giving control of the two pathogen stage.
Materials and methods

1700 micro-organisms (512 fungi, 881 bacteria and 307 yeasts) were isolated from vegetable material coming from untreated vineyards and tested for their capacity of reducing \textit{P. viticola} development. This test was performed using the following method. Leaf disks were put in contact with isolates (grown in a liquid culture) for five minutes and then inoculated with \textit{P. viticola}, leaving them overnight, at 20°C, on a suspension of sporangia. The day after leaf disks were put in humidity chamber, at 20°C and, at the end of disease incubation period, they were observed with light microscope for presence and quantity of sporangia/sporangiophores.

Seventeen bacteria active in the first test, were tested for the ability to reduce or inhibit of sporangia germination of \textit{P. viticola} in co-cultures by means of light microscope observation.

Twenty nine micro-organisms selected also among the active micro-organisms of the first test, were tested specifically for the ability to reduce or inhibit sporangiophore development, by treating infected leaves. \textit{P. viticola} inoculated leaves were treated with the potential BCA (grown in liquid culture) and then incubated at 20°C overnight. One day later leaves were put in humidity chamber at 20°C. At the end of incubation period, the number of sporangiophores was quantified with a light microscope.

From the collection, 73 micro-organisms were randomly chosen and tested during two year for the ability to reduce the primary inoculum: 33 were evaluated in the first year; 40 in the second year, plus 14 resulted effective in the previous year. This test was performed as described by Pertot et al. (2003).

Results and discussion

Out of 1700 organisms applied at infection time of the leaf disks with \textit{P. viticola}, 47 were able to reduced or prevent sporulation (Figure 1). Out of the 22 active bacteria 17 were tested for their capacity to inhibit sporangia germination and seven inhibited this process (Figure 2). Twenty nine out of the 47 active microorganisms were tested for capacity to inhibit sporangia and sporangiophore development from infected leaf disks. Ten showed activity (Figure 3).

Germination of over-wintered oospores in natural conditions was prevented by14 of the 33 in the first year tested micro-organisms, but only one of them was effective in the following year; this raises again the issue of the environmental conditions relevance in influencing BCA efficacy. In the second year four micro-organisms, among the 40 new tested ones, were also effective.

![Figure 1. Potential BCAs tested for global reduction of incidence and/or severity of infections measured as formation of sporangia/sporangiophores on leaf disks](image-url)
Figure 2. Potential BCAs tested in dual culture for inhibition of sporangia germination

Figure 3. Potential BCAs tested for inhibition of sporangiophore development (means separated at $P \leq 0.05$ by Duncan’s test)
Additionally the ability to kill and decompose grape leaves disks was noted. Six fungi, six bacteria and two yeasts were able to rapidly kill and decompose, thus reducing disease on leaf disks.

In conclusion we identified seven bacteria isolates that were able to fully inhibit sporangia germination; four bacteria, four fungi and two yeasts were able to inhibit the formation of sporangiophores. Only one fungus was able to inhibit oospore germination in the trials for two consecutive years. Up to now we have identified 14 micro-organisms showing positive results in biocontrol activity in more than one test. Some preliminary field trials have shown that an unsatisfying disease control is achieved against grapevine downy mildew if only a single BCA is used. In greenhouse and field studies, we are currently testing the use of the effective micro-organisms against *P. viticola* within an integrated approach, in which both the over-wintering inoculum and secondary reproduction are targeted.

Acknowledgements

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References


Disease control on organically grown cyclamen

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Abstract: Results of three years of experimental trials are reported on setting up of biological control strategies for cyclamen, grown under the organic agriculture regulation (REG. CE 2091/91). Particularly, some antagonistic Fusaria showed satisfactory control of Fusarium oxysporum f. sp. cyclaminis and copper sulphate reduced damages caused by Erwinia carotovora subsp. carotovora, while inadequate, for flower plants, was the control of Botrytis cinerea obtained using the first product registered in Italy based on Trichoderma harzianum. Also fertilizers authorized for organic agriculture, not specifically designed for floriculture, caused a reduction of commercial quality of cyclamen. Data collected show that a long research work is still necessary to reach applicable and sustainable solutions for the growers.

Key word: organic floriculture, cyclamen, Fusarium wilt, bacterial soft rot

Introduction

Organic production is one of the most stimulating agricultural sector, from the economic and scientific point of view (Bolkmans, 1999). A strong consumer’s interest is increasing, particularly in northern Europe, in the field of ornamental plants; for this reason, it is necessary to inform the growers on realistic use of organic production strategies applied together to obtain competitive productions. The ornamental plants, in fact, represent a not essential human need and the aesthetic value is the main target to reach, without damage, or pathogen and pest presence. But some problems need to be solved: 1) on the market are still not available nurseries that produce propagative material or young plants, obtained by using the rules of organic production; 2) products for disease control must be available and effective; 3) fertilizers must be designed especially for floriculture, providing needed nutrients to the plants, in accordance with the REG CE 2092/91 (Canali et al., 1999), as cultivation substrates that, possibly, should have a suppressive activity (Garibaldi & Gullino, 1989); 4) the quantity and the quality of productions must be comparable with the “conventional” ones; 5) the pots should be made of biodegradable materials, in order to compost them at the end of the life cycle of the plants. In many cases, experimental trials were carried out to solve specific phytopathological or cultural problems (Jarvis, 1992; Daughtrey et al., 1995; Hausbeck & Moorman, 1996), but it is necessary to face production problems from a more general point of view. This special approach was tried on cyclamen, an economically important flower crop grown as pot plant.

Materials and methods

Three experimental trials, from 1998 to 2001, were carried out at the Centro Regionale di Sperimentazione e Assistenza Agricola of Albenga (Northern Italy) and focusing on the control of key-pathogens for this crop: Fusarium oxysporum f. sp. cyclaminis, Botrytis cinerea and Erwinia carotovora subsp. carotovora. Moreover any agronomic and prophylactic practice useful to reduce disease incidence (fast elimination of infected plants,
cleaning of production sites and pots) and to grow the crop were adopted. The Concerto F1 (Sluis & Groot), grown in a substrate authorized in organic agriculture and specific for cyclamen was used. The normal cultural density used by Albenga growers (10 tetraploid cyclamen plants/m²) was adopted. Experimental trials were designed by using randomised blocks with 4 replicates (15 plant/replicate). Antagonists Fusaria were applied against F. cyclaminis. In the first trial, carried out in 1998, two different cultural conditions were simulated; condition 1: use of antagonistic Fusaria (mixed to the substrate) from the beginning of the cultivation, tested in critical cultural conditions, i.e. used in nursery where normally cyclamen was grown and where benches and pots were uncleaned. Condition 2: production of young plants under greenhouse in clean benches and pots, using conventional fungicides for disease control. From these two nursery conditions, plant were normally grown in open field, under shading screen, where the control of F. cyclaminis was divided in “conventional” and “organic”. Particularly, biological control was applied following instruction described in a previous experimental work (Minuto et al., 1995). The second and the third trial, carried out in 2000 and 2001, were organized using only young plants grown under Condition 2 and, then, cultivated under shading screen. The evaluation of effectiveness of chemical and biological products against B. cinerea and E. carotovora was carried out, during the 2nd and the 3rd trial, both under shadow and under greenhouse conditions, transferring 50% of plants, grown under shading screen, in greenhouse in November. Under “organic” conditions products for insect control and fertilization authorized in organic agriculture were used, and compared with “conventional” products and techniques adopted in the Albenga area (Minuto et al., 2002). The results obtained by adopting organic and conventional disease control and cultivation strategies were compared with those obtained on plants grown organically and not protected against pathogens. A natural pathogen infection, placing one plant infected by F. cyclaminis, B. cinerea and E. carotovora in each plot was simulated. The percentage of infected and dead plants was calculated and the final quality of the production, following the commercial parameters used in the Albenga area was evaluated. Temperature and relative humidity under greenhouse and under shading screen were measured. All data were analysed by following Duncan’s multiple range test (P≤0.05).

Results and discussion

Disease control measures in nursery were very important for the control of Fusarium wilt. Particularly, the effectiveness of application of biological and chemical control measures is consistent only when healthy propagative material grown in a clean environment is used (Tables 1 and 2). Antagonistic Fusarium applied at the start of the cultivation, also under condition 1, provided an interesting disease control, higher than that obtained by using benomyl. The use of Trichoderma harzianum and copper sulphate reduced, in the second and third trial, disease incidence of B. cinerea, even if their activity was inconsistent, compared with that of chemicals (Table 3). This result could be a consequence of the variability of environmental conditions: frequent and intensive rainfall induced a high disease incidence under shade in the autumn 2001, comparable with disease incidence observed under greenhouse, where humidity constantly reached saturation. Under greenhouse, these environmental conditions caused also a reduction of efficacy of chemicals, probably due to the fast diffusion of the pathogen. A copper based product both in “conventional” and in “organic” cultivation controlled E. carotovora (Table 4). At the end of the second and the of third trial the evaluation of the plants quality was carried out. The best results were obtained growing cyclamen under “conventional” techniques; particularly, it was observed a higher flower production and a higher production of first quality plants (Table 5). Under organic agriculture conditions, the poor quality of plants was caused also by the kind of fertilisers used, not specifically designed for floriculture, rich in nitrogen and poor in phosphorus and potassium, characterised, also, by a very low content of these last nutrients. For organic ornamental production, also biological control products are not always designed to solve all phytopathological problems. For this reason, it is very important to adopt any cultural
technique (i.e. reduced plant density, farm cleaning, healthy propagative material, climate control under greenhouse), that can create conditions unfavourable for the spread of disease. Biological products in nurseries, potentially infected by \( F. \) cyclaminis, are not able to stop initial infections on plants; therefore the final production is commercially unacceptable.

Table 1. Control strategies adopted in 1998 on cyclamen

<table>
<thead>
<tr>
<th>Control strategy in nursery</th>
<th>Product applied against</th>
<th>Control strategy in farm</th>
<th>F. cyclaminis</th>
<th>B. cinerea</th>
<th>E. carotovora</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Condition 1:</strong> Organic</td>
<td>Trichoderma harzianum alternate with copper sulphate</td>
<td>organic F.O. 251/2 Rben°</td>
<td>F.O. 251/2 Rben°</td>
<td>copper sulphate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alternation of copper sulphate, clorothalonil, iprodione and dithianon</td>
<td>conventional benomyl°°</td>
<td>conventional benomyl°°</td>
<td>copper sulphate</td>
<td></td>
</tr>
</tbody>
</table>

**Condition 2:** Conventional Benomyl

<table>
<thead>
<tr>
<th>Product applied against</th>
<th>Control strategy in farm</th>
<th>F. cyclaminis</th>
<th>B. cinerea</th>
<th>E. carotovora</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichoderma harzianum alternate with copper sulphate</td>
<td>organic F.O. 251/2 Rben°</td>
<td>F.O. 251/2 Rben°</td>
<td>copper sulphate</td>
<td></td>
</tr>
<tr>
<td>Alternation of copper sulphate, clorothalonil, iprodione and dithianon</td>
<td>conventional benomyl°°</td>
<td>conventional benomyl°°</td>
<td>copper sulphate</td>
<td></td>
</tr>
</tbody>
</table>

° Roots immersion and soil mixing at transplanting in farm, °° Soil mixing

Table 2. Effect of different control strategies against \( Fusarium \) wilt of cyclamen on plants grown following nursery condition 1 and nursery condition 2 (Albenga, 1998)

<table>
<thead>
<tr>
<th>Control strategy in farm</th>
<th>Product</th>
<th>Dead plants at days after transplanting (%)</th>
<th>26</th>
<th>66</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>50 b*</td>
<td>0  a</td>
<td>70 b</td>
</tr>
<tr>
<td>Organic</td>
<td>F.O. 251/2 Rben°</td>
<td>0 a</td>
<td>14 a</td>
<td>7 a</td>
</tr>
<tr>
<td>Conventional</td>
<td>Benomyl°°</td>
<td>4 a</td>
<td>16 a</td>
<td>7 a</td>
</tr>
</tbody>
</table>

*Values followed by the same letter do not significantly differ following Duncan’s multiple range test \((P\leq0.05); ° Roots immersion and soil mixing at transplanting in farm, °° Soil mixing

This situation could create severe problems to the growers, particularly on cyclamen, distributed by few, big nurseries highly specialized that cannot be considered completely immune by infections of \( F. \) cyclaminis. In these conditions, considering that the infections of cyclamen seeds can be controlled only with the use of systemic chemicals and considering the present small dimension of the market of the production of organic young plants for potted flowers, it is difficult for conventional nurseries to set specific areas especially designed for organic productions.

Table 3. Effect of control strategies for the control of \( B. \) cinerea (Albenga, 2000 and 2001)
### Table 4. Effect of copper sulphate for the control of *E. carotovora* (Albenga, 2001)

<table>
<thead>
<tr>
<th>Control strategy in farm</th>
<th>Product</th>
<th>Infected plants (%)</th>
<th>2000</th>
<th>2001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>shading</td>
<td>greenhouse</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td></td>
<td>0.0 a*</td>
<td>21.3 c</td>
</tr>
<tr>
<td>Organic</td>
<td><em>Trichoderma harzianum</em> alternate of with copper sulphate</td>
<td></td>
<td>0.0 a</td>
<td>10.3 b</td>
</tr>
<tr>
<td>Conventional</td>
<td>Alternate of copper sulphate, chlorotalonil, iprodione and ditianon</td>
<td></td>
<td>0.0 a</td>
<td>1.2 a</td>
</tr>
</tbody>
</table>

*Values followed by the same letter do not significantly differ following Duncan’s multiple range test (*P* ≤ 0.05)*

### Table 5. Final plant quality grown under organic and conventional cultivation systems (Albenga, 2001).

<table>
<thead>
<tr>
<th>Applied control strategies</th>
<th>flowers/plant (no)</th>
<th>1st choice plants (%): 2000</th>
<th>2nd choice plants (%): 2000</th>
<th>No commercial plants (%): 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>21.4 b *</td>
<td>0.0 b</td>
<td>55.0 a</td>
<td>45.0 b</td>
</tr>
<tr>
<td>Organic</td>
<td>19.4 b</td>
<td>0.0 b</td>
<td>45.0 a</td>
<td>55.0 b</td>
</tr>
<tr>
<td>Conventional</td>
<td>28.4 a</td>
<td>25.3 a</td>
<td>50.0 a</td>
<td>24.7 a</td>
</tr>
</tbody>
</table>

*Values followed by the same letter do not significantly differ following Duncan’s multiple range test (*P* ≤ 0.05)*

On the contrary, the crop management it is easier using plants originating from nurseries that applied conventional cropping systems with chemical control of *Fusarium* wilt. In these conditions, the use of antagonistic *Fusaria* during the crop guaranteed a good protection level; their correct use gave results comparables with those achievable using benzimidazoles (Garibaldi and Minuto, 1993). The copper based products showed good control against *E. carotovora*, both under “conventional” and “organic” cultivation strategies, while, against *B. cinerea*, showed reduced activity when used under “organic” conditions. In conclusion, these results demonstrate that development of organic cultivation strategies in floriculture is at the beginning and results are still unsatisfactory, considering the final quality of plants.

### Acknowledgements

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Integration of the use of the antagonist *Ulocladium atrum* in management of strawberry grey mould (*Botrytis cinerea*)

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Abstract: *Botrytis cinerea* is causing fruit rot of strawberry after infection of flowers and fruits by airborne conidia. The antagonist *Ulocladium atrum* has been selected for its ability to compete with *Botrytis* spp. in necrotic tissues. Antagonism results in suppression of sporulation of the pathogen and in blocking growth of *B. cinerea* from necrotic plant tissue to adjacent healthy tissue. Preliminary experiments in strawberry demonstrated the potential of *U. atrum* to suppress *B. cinerea* sporulation on strawberry leaves as well as to protect flowers from invasion by the pathogen. A series of ten field experiments has been carried out with annual waiting-bed crops between 1996 and 2003 to optimise and to integrate the use of *U. atrum* in control of grey mould in such a strawberry cropping system. First experiments indicated that applications before flowering did not increase disease control and that regular applications twice weekly during flowering were superior to applications once weekly. In 2002 and 2003, a decision support system was used to optimise timing of antagonist applications and to avoid applications under conditions not favourable for disease development. In comparison with regular applications of *U. atrum* twice weekly, applications according to DSS (>7.5% infection chance) were less frequent resulting in slightly weaker disease control (2002; no statistically significant difference) or timed differently resulting in increased disease control (2003).

Key words: strawberry, *Botrytis cinerea*, grey mould, biological control, *Ulocladium atrum*, Decision Support Systems

Introduction

*Botrytis cinerea* is causing fruit rot of strawberry after infection of flowers and fruits by airborne conidia (Jarvis, 1962). Conidia of *B. cinerea* can be produced on necrotic tissues such as necrotic leaves of the strawberry crop but also on various other necrotic plant tissues inside and outside the crop (Braun & Sutton, 1987). The antagonist *Ulocladium atrum* has been selected for its ability to compete with *Botrytis* spp. in necrotic tissues under a broad range of environmental conditions (Köhl, 2001). Antagonism results in suppression of sporulation of the pathogen, as shown in field experiments in onions (Köhl et al., 2003), and in blocking growth of *B. cinerea* from necrotic plant tissue to adjacent healthy tissue, as demonstrated in cyclamen under commercial conditions (Köhl et al., 2000).

Preliminary experiments in strawberry demonstrated the potential of *U. atrum* to suppress *B. cinerea* sporulation on strawberry leaves as well as to protect flowers from invasion by the pathogen. The antagonist did not affect pollination, fruit formation and quality. Based on these results, a series of ten field experiments has been carried out between 1996 and 2003 to optimise and to integrate the use of *U. atrum* in control of grey mould in annual strawberry crops.
**Material and methods**

All field experiments were conducted with cold-stored waiting-bed transplants planted in spring at Wageningen or Horst, The Netherlands. Experiments were carried out in a randomised block design with four to six replicates per treatment with plots planted with 40 to 80 plants. Plots were separated by buffer strips not planted with strawberries.

Inoculum of *U. atrum* was produced on oats (Köh et al., 2000). Conidial suspensions, adjusted at 2x10^6 conidia per ml containing 0.01 % Tween 80, and fungicides were applied with a knapsack sprayer.

Infection chances were forecasted using the decision support system (DSS) Optibol developed by Opticrop BV (Zevenhuizen, The Netherlands).

**Results and discussion**

First experiments were conducted to identify the relevant targets for the antagonist in the crop. Crop sanitation by removal of necrotic leaflets twice weekly during the whole growing season did not result in prevention of grey mould (Figure 1). Weekly application of *U. atrum* before flowering on the transplants followed by twice weekly application during flowering did not result in better disease control than twice weekly applications only during flowering. In the same experiment, applications of *U. atrum* or fungicides during flowering reduced grey mould incidence significantly by 33 % and 57 %, respectively.

In conclusion, necrotic strawberry leaves were no significant inoculum source of *B. cinerea* in the annual strawberry crops planted with waiting-bed plants. This was confirmed by additional observations of aerial spore loads. No differences in spore load above and outside strawberry plots could be detected (Boff et al., 2001).

![Figure 1. Effect of crop sanitation (removal of necrotic leaflets twice weekly), application of fungicides (six treatments with tolyfluanide or iprodione in alternation) and application of *U. atrum* (2x10^6 conidia per ml) on percentage grey mould at harvest. *U. atrum* was applied weekly from transplanting and twice weekly from first green bud appearance (U.a. transplant. + flowering) or twice weekly from first open flowers (U.a. flowering).](image-url)

The results of subsequent field experiments demonstrated that intervals between antagonist applications during flowering must be short to guarantee that newly opened flowers are reached by the antagonist. When a regular application regime was applied, at least two applications per week were found to be necessary (Figure 2).
In 2002 and 2003, field experiments were carried out using the decision support system Optibol (DSS) to optimise timing of antagonist applications and to avoid applications under conditions not favourable for disease development. In 2002, 8 applications of *U. atrum* applied twice weekly gave 50% disease control, whereas 4 applications timed according to DSS gave 30% disease control (data not shown). In the same experiment, 6 applications of Euparene, timed as in practise, reduced grey mould by 93%. In 2003, 7 applications of *U. atrum* at regular intervals of 3 - 4 days resulted in a reduction of grey mould incidence by 24% (Figure 3). When applications were timed according to the DSS (at an infection chance >7.5%), 6 applications were carried out resulting in a reduction of grey mould incidence by 39%. Applications of fungicide as timed in practise and at regular intervals adjusted for rainfall, resulted in the same control level. Fungicide application timed following the DSS rules reduced grey mould incidence by 67%.

Results of field experiments indicate that a moderate control of grey mould can be achieved with multiple applications of the antagonist *U. atrum*. Using a decision support system to optimise timing of antagonist applications during flowering tended to result in less and/or more effective applications. This finding has to be confirmed in further experiments. In these experiments, the integrated use of the antagonist and fungicides will also be assessed,
e.g. with applications of a fungicide if the forecasted infection chance is high with >25 %, and antagonist application if the forecasted infection chance is moderate with > 7.5 % - 25 %.

In most experiments, symptomless harvested strawberries were incubated for assessment of post-harvest grey mould. Percentages of fruits with post-harvest grey mould varied considerable amongst experiments. Fungicides had inconsistent control effects. Antagonist applications did not reduce post-harvest grey mould in most experiments.

Epidemics of powdery mildew occurred in most experiments in the strawberry crops resulting in leaf infections. Generally, fungicide treatments reduced powdery mildew significantly whereas *U. atrum* treatments did not affect this disease.

Yields of strawberries belonging to the first quality class were significantly increased after fungicide treatments compared to the untreated control. *U. atrum* treatments usually resulted in yields comparable to the untreated control. This may be due to the differential effect of treatments in powdery mildew control. Further research is needed to combine grey mould control by *U. atrum* with powdery mildew control by other means.

**Acknowledgements**

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Suppression of *Rhizoctonia* root rot of tomato by *Glomus mosseae* BEG12 and *Pseudomonas fluorescens* A6RI is associated with combined modes of action

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Root-rot due to *Rhizoctonia solani* is a major soilborne disease causing growth and yield depression. The ability of *Glomus mosseae* BEG12 and of *Pseudomonas fluorescens* A6RI to suppress this soilborne disease in tomato was assessed by comparing the shoot and root growth of infested plants when protected or not by these beneficial strains. Disease suppression of soilborne diseases is commonly ascribed to both reduction of the epiphytic (microbial antagonism) and parasitic (induced resistance) growth of the pathogen. The pathogen growth at the root surface and in the root tissues was then assessed by microscopic observations. The root architecture of the tomato plants in the different experimental conditions has been further characterized by measuring total root length, mean root diameter, number of root tips and by calculating root branching degree. Data obtained indicate that both beneficial strains suppressed root-rot due to *R. solani*. This suppression was related for both organisms to microbial antagonism and induced resistance. On the top of these modes of actions, the increase of the root length and number of root tips appeared to also contribute to the disease suppression.
Biological control of *Pythium aphanidermatum* in cucumber with combined applications of bacterial antagonists with chitosan

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**Abstract:** *Pythium aphanidermatum* (Edson) Fitzp. is an important disease in greenhouse vegetables grown on soilless substrate systems, especially if the nutrient solution is recirculated. The disease is difficult to control. In the short term, there are no prospects for breeding resistant cultivars, and fungicides (i.e. propamocarb and metalaxyl) are only effective if used as preventive applications. Used rockwool slabs can have disease suppressive properties due to the autochthonous microflora, but this is not always a suitable solution due to the risk of other diseases. Many years of research on biological control agents has resulted in several fungal and bacterial isolates with biocontrol properties, but their effect is often insufficient or not reproducible. With the purpose to obtain more reliable biocontrol agents, many bacteria have been collected from suppressive rockwool. Their antagonistic properties were tested in small bioassays. The most effective organisms occurred to be *Lysobacter enzymogenes* isolate 3.1T8 originating from young roots, and a filamentous actinomycetes *Streptomyces* sp. isolate B65 originating from old roots in used rockwool slabs. Nevertheless, larger scale experiments with these isolates in an ebb and flow systems with recirculated nutrient solution were disappointing. Therefore, combinations of the two bacteria with chitosan, which is known as an elicitor, were tested. The combination of two bacteria alone did not give any biocontrol effect. But all combinations between the two bacteria and chitosan were highly effective; the disease suppression was 55-95% as compared to the control with only *P. aphanidermatum*. This synergistic effect between the antagonistic bacteria and a natural product, opens new possibilities for the development of a more reliable biocontrol strategy for the control of *P. aphanidermatum*.

**Key words:** biological control, Pythium stem and root rot, *Lysobacter enzymogenes*, *Streptomyces* sp., chitosan, synergistic effect

**Introduction**

*Pythium aphanidermatum* (Edson) Fitzp. is an aggressive and economically important pathogen in greenhouse-grown cucumbers. Especially in substrates like rockwool which exhibit high water retention capacity, it can flourish and spread rapidly due to the production of zoospores. The pathogen causes severe stem and root rot resulting in wilting and dying plants. Control is difficult, since resistant cultivars do not exist and fungicides (i.e. propamocarb and metalaxyl) have to be applied preventively and should be avoided as much as possible for environmental reasons.

Several antagonistic micro-organisms such as *Pseudomonas* spp., *Streptomyces* spp., *Trichoderma* spp., *Bacillus* spp., and *Pythium oligandrum* have the potential to control *P. aphanidermatum*. However, effects of biocontrol agents in soilless systems often were insufficient under practical conditions or difficult to reproduce (Paulitz & Bélanger, 2001; Postma et al., 2001). To overcome this problem, the combination of biological control agents with different mode of actions might be more effective and reliable at controlling *Pythium* than single strains. In previous research projects the following bacterial isolates and one
compound have been found to be promising as (bio)control agent of *P. aphanidermatum*. The modes of actions are suggested to be different.

*Lysobacter enzymogenes* isolate 3.1T8 is a gram-negative bacterium originating from young cucumber roots (Folman et al., 2003b). This isolate showed effective control of *P. aphanidermatum* in short bioassays and is expected to compete with *Pythium* for infection sites on the root (Folman et al., 2003a). *Streptomyces* sp. isolate B65, a filamentous actinomycete originating from old roots in used disease-suppressive rockwool slabs (Postma et al., 2001). This isolate is expected to control *Pythium* by colonising dead root material, and as a consequence decreasing the inoculum production by *Pythium*. Chitosan has been described as inducer of host defense responses (El Ghaouth et al., 1994; Benhamou et al., 1998).

In the present research, combinations of one, two, or three of the above mentioned control agents have been tested for there effect on *P. aphanidermatum* in cucumber plants grown on rockwool blocks in several independent greenhouse experiments.

**Material and methods**

**Antagonists**

*Lysobacter enzymogenes* isolate 3.1T8 was originally isolated from root tips of cucumber plants in the generative stage (Folman et al., 2003b). *Streptomyces* sp. isolate B65 had been isolated from dead root material taken from used disease suppressive rockwool slabs (Postma et al., 2001). Both bacterial antagonists showed strong *in vitro* inhibition of *P. aphanidermatum* in dual culture and were able to control *Pythium* in small scale bioassays. The applied concentrations were between 5x10⁷ and 2x10⁸ CFU per plant, equivalent to 10⁵ and 4x10⁶ CFU per ml nutrient solution.

**Chitosan**

Crap-shell chitosan was solved as described by El Ghaouth et al. (1994). Applied dosages were 0.1 or 0.4 g chitosan per plant

**Greenhouse experiments**

Experiments were conducted in ebb-and-flood systems with a recirculated nutrient solution, as described in Postma et al. (2001). Each treatment consisted of four independent ebb-and-flood systems, each containing 10-12 cucumber plants. Antagonists and chitosan were applied 5 days after sowing, when seeds had just germinated. *P. aphanidermatum* grown in liquid V8 medium was applied two days later (approx. 10⁵ oospores per plant). Disease development was scored regularly during 4 to 5 weeks. Greenhouse temperature was 25/20°C (day/night).

**Results**

The experiments with different combinations of (bio)control agents showed that all combinations of *Lysobacter* and *Streptomyces* together with chitosan were very effective in controlling *Pythium* (Table 1). Bacterial antagonists alone or combined with each other were not effective. Chitosan alone resulted in a delayed disease development, but was not effective enough to keep a low disease level up to the end of the experiments.

Three other experiments showed that the positive effect of *Lysobacter* with chitosan could be replicated, even at higher greenhouse temperature (temperature peaks of 35°C) or at a lower chitosan dosage (0.1 g per plant in stead of 0.4 g) (Figure 1). Only in experiment four, *Lysobacter* with the lower dosage of chitosan was not effective.
Table 1. Percentage disease reduction in cucumber plants inoculated with *Pythium aphanidermatum* due to different combinations of (bio)control agents

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysobacter</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td>Streptomyces</td>
<td>13</td>
<td>–</td>
</tr>
<tr>
<td>Chitosan</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Lysobacter + Streptomyces</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Lysobacter + chitosan</td>
<td>–</td>
<td>95</td>
</tr>
<tr>
<td>Streptomyces + chitosan</td>
<td>–</td>
<td>90</td>
</tr>
<tr>
<td>Lysobacter + Streptomyces + chitosan</td>
<td>62</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 1. Percentage diseased cucumber plants due to the inoculation with *Pythium aphanidermatum* in five independent experiments carried out in an ebb-and-flood system. Treatments are: *Pythium* alone, *Pythium* with the antagonist *Lysobacter enzymogenes* 3.1T8 and 0.4 or 0.1 g chitosan per plant.

**Discussion**

As hypothesised, the combination of different (bio)control agents was much more effective than the separate applications. A clear synergistic effect was found due to combined application of *L. enzymogenes* isolate 3.1T8 or *Streptomyces* sp. isolate B65 together with chitosan.

The mode of action of this combined application is not known. The synergistic effect of chitosan can be due to (1) stimulation of the antagonist, (2) inhibition of the pathogen, or (3) immunisation of the plants. Dual culture tests showed that inhibition zones between the antagonist and the pathogen were similar on agar with and without chitosan, indication that chitosan is not stimulating antibiotic production.

For the first time after many years of research, we now have a biocontrol agent with strong and reproducible effects on *P. aphanidermatum* in cucumber. Disease control even lasted under relatively high temperatures (35°C), which can occur in greenhouses during
summer time. These results will be validated as soon as possible in greenhouse experiments with cucumber production under practical conditions.

Acknowledgements

This research is supported by the Dutch Ministry of Agriculture, Nature and Food quality (DWK programme 397) and the Dutch Ministry of Economic Affairs (project EETK02009). The bacterial strain *Lysobacter enzymogenes* 3.1T8 was isolated previously in a project funded by the Dutch Technology Foundation (STW) (project LBI.3679).

References


Evaluation of the sustainability of strategies that include biocontrol agents to reduce chemical residues on strawberry fruits

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Abstract: Biocontrol agents (BCAs) have become one of the most promising tools to be developed for reducing the use of chemical pesticides both against insects and diseases. Despite some important benefits, the use of BCAs has some drawbacks, which prevent large commercial diffusion. There is increasing agreement among researchers about the necessity of better common understanding of BCAs implications. A sustainability approach could be very useful in this direction allowing us to highlight economic, environmental and social aspects of BCAs utilisation. This study focuses on the development of a sustainability framework for the selection of the best combinations of BCAs in integrated strategies to reduce chemical input, after evaluating all the important factors, including economical, social and environmental ones. The data are obtained from an integrated pest management (IPM) experiment in growing strawberries in the Trentino Region. The different techniques are described and costs and yield losses from one-year experiment are compared with average costs and yield losses calculated as average for that area. Data on pesticides residues are also introduced in the assessment framework. Marketing organisation, market conditions and social acceptability are considered in order to highlight necessary conditions for a successful biological control from a sustainability point of view.

Key words: biocontrol, IPM, sustainability, strawberry

Introduction

Biocontrol agents (BCAs) have become one of the most promising tools to be developed for reducing the use of chemical pesticides both against insects and diseases. Although excellent research has clearly demonstrated their potential against specific diseases in controlled experiments, the wide-scale use of biocontrol has not been adopted in commercial production systems (Ellis, 2003). The reasons may be divided into two blocks: reasons that are common to other integrated pest management (IPM) strategies and reasons that are specific for biological control methods. Among the former, Aitken et al. (1995) highlighted the relevance of poor communication and lack of teamwork in determining the failure of IPM projects. Among the latter it is necessary to mention some drawbacks generally associated to the use of BCAs, that is higher cost of treatments, higher yield losses, shorter shelf life and possible non-target effects.

In order to properly evaluate success or failure of BCAs, benefits and drawbacks should be reconducted into the same assessment framework. In particular, we share the conviction that evaluation should go beyond technical efficacy (reduction of pest population) and take into account other factors such as “practical efficacy”, “commercial viability”, “sustainability” (the term sustainability is used in a quite restrictive meaning. It is used as the capacity of the BCAs to survive in the environment and continue to be effective over time) and “providing public benefits” (Gelernter & Lomer, 2000).
In this direction the sustainability framework as developed after Brundtland Report (1987) might be very useful. Beyond the innumerable different definitions presented in the economic literature, it is commonly recognised that the sustainability concept involves at least three dimensions: the economic, the environmental and the social one.

In this perspective the study aims at assessing different IPM strategies and BCAs combinations for protecting strawberry cultivations in Trentino Region, according to the sustainability framework. Strawberries are particularly interesting because “they are world-wide produced with practices that many consider unsustainable” (Pritts, 2000) and are included in the “list of top-ten foods to avoid because of high pesticide residues” (Ames et al., 2003). The chosen study area (Valsugana) is interesting because it produces more than half the production of the entire province (1,790 tonnes of strawberries in 2002). In that area operates the most important Italian Association of small fruits, APA Sant’Orsola, a cooperative that aggregates about 1,500 small growers producing different types of berries. “Sant’Orsola” is the leading brand in Italy for small fruits and shows great attention to the safety of its production forcing all its associates to adopt a quite restrictive protocol on IPM.

Material and methods

The SafeCrop Centre conducted a two-year (2002-2003) field experiment on two strawberry farms associated to Sant’Orsola cooperative. The natural situation of the two sites (Canezza and Cirè) is quite similar and also cultivation conditions are the same (variety Elsanta cultivated soil less under tunnel). Under these conditions the most dangerous fungal disease remains powdery mildew (*Sphaerotheca macularis*). Ten different strategies (plus an untreated strategy) were experimented on the two sites according to a randomised blocks design with three replications. The ten strategies are differentiated by the active principles used whereas the number of treatments (7) and the treatment timing (every 7 days) were the same. Among these strategies, three groups of strategies are identified: a) four IPM strategies (1, 2, 4, 7); b) three IPM strategies integrated with BCAs (3, 5, 6) and c) three strategies based solely on BCAs (8, 9, 10).

Focusing on the three IPM strategy integrated with BCAs (Table 1), it is useful to highlight the difference in terms of reduction in the number of conventional treatments and the sequence of treatment. In particular, strategy 5 and 6 include only two chemical treatments whereas in strategy 3 more than half the treatments are chemical. Strategy 6 is the only one, which guarantees 4 weeks before harvest without chemical inputs.

<table>
<thead>
<tr>
<th>Strategies</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 IPM 1BCA</td>
<td>Azoxy.</td>
<td>Sulphur</td>
<td>Penconaz</td>
<td>Azoxy.</td>
<td>AQ10</td>
<td>AQ10</td>
<td>AQ10</td>
</tr>
<tr>
<td>5 IPM 2BCA</td>
<td>Azoxy.</td>
<td>Trichodex</td>
<td>Trichodex</td>
<td>Trichodex</td>
<td>Trichodex</td>
<td>Azoxy.</td>
<td>AQ10</td>
</tr>
<tr>
<td>6 IPM 2BCA+ Azoxy.</td>
<td>Penconaz</td>
<td>Trichodex</td>
<td>Trichodex</td>
<td>Trichodex</td>
<td>Azoxy.</td>
<td>AQ10</td>
<td>AQ10</td>
</tr>
</tbody>
</table>

According to the sustainability aim of this contribution, only 3 vectors out of the entire data set collected in the experiment conducted in 2003 are considered here: the quantity of treatments against powdery mildew, the incidence of fruit losses (yield loss) and the pesticides residues (azoxystrobin) found on fruit at the harvest time. Fruits were also analysed for penconazole residues but the principle was no detectable in any cases.
Having collected the market price of the fungicides used in the experiment, it was possible to calculate the cost of treatments for each strategy and the average cost of fungicide treatments currently suggested by the extension service to strawberry growers located in that area. By interviewing some technicians of the extension services we also estimated the average yield loss accepted as normal for this type of cultivation.

**Results and discussion**

According to the sustainability framework, the first aspect to discuss is the economic one. We have first to look at the difference in terms of revenues and costs. From the revenue side, we need to restrict our analysis to the quantity losses because of the current impossibility to quantify a price premium for strawberries obtained with IPM integrated with BCAs.

From the cost side it is quite easy to calculate the cost of strategies in terms of treatments. Figure 1 graphically represents the 11 strategies in relation to three dimensions: average loss, costs of treatments and presence of residues (azoxystrobin) for the most interesting site (Cirè).

![Figure 1. Some results from BCAs experiment in Cirè (2003)](image)

Considering as benchmark the estimated average cost of treatments (38.41 Euros/1000 m²) and an average yield loss equal to 5% (Figure 1a), we realise that only strategy 1, 2 and 3 are included in the feasible region. Strategy 3 with one BCAs guarantees goods results from the economic point of view but it still presents residues at the harvest time.

If we quantify the difference in term of time spent applying the fungicides (1 hour for each treatment to a cost of 6.5 Euros), the total cost of treatments rises dramatically. Figure 1b shows what happens if we move the reference line to 116.42 Euros which is the average total cost for 12 treatments. As a result of the different number of treatments (7 in the experiment against the 12 normally performed), strategy 6 becomes very interesting both from an economic and an environmental point of view. In fact it falls within the new feasible region and does not present detectable residues. Moreover strategy 6 guarantees the absence of chemical inputs in the four weeks before harvest and therefore it is the best strategy for the
consumers’ safety. Therefore we can define this strategy 6 as fully sustainable, at least in cases with the same conditions registered in Cirè.

In fact, in Canezza’s experiment, strategy 6 lies below the benchmark line of total cost but it shows a yield loss equal to 8.33%, therefore higher than the average acceptable one. This does not automatically mean that this strategy is unsustainable but it points out one of the most important conditions for sustainability of BCAs strategies: the valorisation of the product safety. Given the increasing willingness to pay for safer products, it becomes fundamental to find differentiation mechanisms that guarantee a premium price to safer products and counterbalance the higher yield loss. In the case of Sant’Orsola cooperative, which already benefits from a leader position in the market, it should not be too difficult to reinforce its safety policy in order to assure a premium price to producers. Moreover it has been already demonstrated in the literature that vertical integration leads to less final residues (Kilmer et al., 2001) and that the conviction of the leader inside an organisation is fundamental for the success of IPM projects (Gelernter & Lomer, 2000).

Finally it is important to stress that a reduction in the use of chemical inputs through BCAs implies not only safer products for consumers but also higher safety at work for growers and, ultimately, a better quality of life for residents. Therefore a higher environmental sustainability leads also to social sustainability by letting strawberry growers to regain part of the lost social legitimacy to produce.

The results of this experiment allows us conclude that a sustainability approach might shed new light on the understanding of BCAs implications. Differentiation mechanism and premium price play a key role in order to internalise increased environmental sustainability and to guarantee economic sustainability. Wider interdisciplinary research and extensive open-field experimentation are still necessary.

References


Use of biocontrol agents against powdery mildew in integrated strategies for reducing pesticide residues on strawberry: evaluation of efficacy and side effects

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Abstract: Pesticide residues became a major issue that resulted in legislative actions that limit and regulate pesticide use. Nevertheless, consumers and policy makers are demanding lower pesticide input to increase quality and safety of food along the whole food chain, without increasing costs to farmers and consumers. Strawberry powdery mildew (Sphaerotheca macularis f.sp. fragariae) is a serious disease of strawberry in warm and dry climates or in greenhouses. At least six-seven treatments are required for each growing cycle in off-soil strawberry plantation in tunnels in northern Italy environments. The aim of this work was to evaluate the efficacy of alternatives to chemical pesticides and their integration into strategies against powdery mildew of strawberry with the aim of reducing pesticide residues on fruits. Side effects on mites were also evaluated. Biocontrol agents (BCAs), like Ampelomyces quisqualis, Bacillus subtilis and Trichoderma harzianum T39, can control the disease, but to a lesser extent as compared chemicals, and their level of activity varies according to the location, environment and time of application. When BCAs were applied in combinations with chemicals a good reduction of residues was achieved while maintaining the same control efficacy against. All the proposed strategies did not increase Tetranychus urticae, they had no side effects on Amblyseius andersoni populations and consequently they can be regarded as safe for the ecological balance in strawberry greenhouses.

Key words: Sphaerotheca macularis, powdery mildew, strawberry, biocontrol, side effects

Introduction

Pesticide residues became a major issue that resulted in legislative actions that limit and regulate pesticide use. The upper legal limits of a pesticide residue to be found on a food (maximum residue level, MRL) have been fixed in all countries. The MRL is not a toxicological limit and its violation is not necessarily a cause of concern for public health. In fact for authorized pesticides, the MRLs are set at the maximum safe level that one would expect if the pesticide is used according to the rules and restrictions specified in the authorisation. However, consumers and policy makers are demanding lower pesticide input to increase quality and safety of food along the entire food chain, without increasing costs to farmers and consumers.

Strawberry powdery mildew (Sphaerotheca macularis f.sp. fragariae) is a serious disease of strawberry in warm and dry climates or in greenhouses (Mass, 1998). Soil-less production in greenhouses have several positive effects on fruit quality and post-harvest keeping and they allow expanding the production period. They also help in the control of
several important diseases like grey mould, anthracnose or root rots. However, without the inhibitory effect of rain on conidia, they result in an increase of powdery mildew incidence (Pertot et al., 2001; Xiao et al., 2001). *S. macularis* is controlled primarily by chemical treatments. At least six-seven treatments are required for each growing cycle in soil-less plantation in tunnels under northern Italy conditions.

The purpose of this work was to evaluate the efficacy of alternatives to chemical pesticides and their integration into strategies against powdery mildew of strawberry with the aim of reducing pesticide residues on fruits. The influence of the proposed strategies on the tetranychid mite pest and predatory mites was also evaluated. The most widely used fungicides against powdery mildew on strawberry were compared and alternatives (biocontrol agents) were tested for efficacy in controlling *S. macularis* in experimental field trials.

### Materials and methods

#### Field trials

Trials were performed in 2003, in two locations in Trentino (Cirè and Canezza), on the soil-less most widely used strawberry cultivar (Elsanta), which is highly susceptible to the disease. Test strategies, reference strategies and untreated control were arranged in fully randomised blocks, three replicates per treatment. Plot size was of 24 potted plants, grown on peat. Treatments (Table 1) were weekly applied according to different strategies (Table 2) and environmental data were recorded (temperature and relative humidity). Assessments were carried out weekly, scoring 50 leaves per plot and at harvest time, scoring 20 fruits per plot. For each plot the percentage of infected leaf area and the number of infected fruits were assessed. Disease severity and incidence were calculated both on leaves and on fruits. Statistical analyses of the data were performed using the SPSS software. Means were separated using Duncan’s test at $P \leq 0.05$. Residues of chemical pesticides in fruits were evaluated at harvest time.

#### Table 1. Product used in the trial

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Commercial name</th>
<th>a.i. (%)</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A azauxystrobin</td>
<td>Ortiva</td>
<td>23,2</td>
<td>0.80 ml/l</td>
</tr>
<tr>
<td>B penconazole</td>
<td>Topas</td>
<td>10,5</td>
<td>0.40 ml/l</td>
</tr>
<tr>
<td>C sulphur</td>
<td>Tiovit</td>
<td>80</td>
<td>3.00 g/l</td>
</tr>
<tr>
<td>D <em>Trichoderma harzianum</em> T39 pinolene</td>
<td>Trichodex + Vaporgard</td>
<td>6</td>
<td>4.00 g/l</td>
</tr>
<tr>
<td>E <em>Ampelomyces quisqualis</em> pinolene</td>
<td>AQ10+ Vaporgard</td>
<td>58</td>
<td>0.08 g/l</td>
</tr>
<tr>
<td>F sodium bicarbonate</td>
<td>SIGMA</td>
<td>99,5</td>
<td>2.00 g/l</td>
</tr>
<tr>
<td>G sulphur</td>
<td>Heliosoufre</td>
<td>51</td>
<td>1.50 ml/l</td>
</tr>
<tr>
<td>H <em>Bacillus subtilis</em></td>
<td>Serenade</td>
<td></td>
<td>4.00 g/l</td>
</tr>
<tr>
<td>I fenarimol</td>
<td>Rubigan</td>
<td></td>
<td>0.30 ml/l</td>
</tr>
</tbody>
</table>

**Untreated control**

#### Side effects on mites in the field trials

Between 15 and 30 adult females of *Tetranychus urticae* were introduced on one leaf per plant, on the six central plants of each plot. One week after *T. urticae* introduction, the same
number of *Amblyseius andersoni* adult females was introduced on the same leaves. Assessments were carried out weekly, scoring 10 leaves (six leaves where mites have been introduced plus four randomly chosen leaves) before and after fungicide or BCA treatments.

Table 2. Sequence of treatments in the applied strategies

<table>
<thead>
<tr>
<th>Strategy no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>B</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>C</td>
<td>B</td>
<td>A</td>
<td>E</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td>B</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>7</td>
<td>A</td>
<td>B</td>
<td>G</td>
<td>G</td>
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<tr>
<td>8</td>
<td>D</td>
<td>D</td>
<td>D</td>
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<td>9</td>
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Results

The development of the disease was similar in the two locations, even if in Cirè, due to the higher temperatures, development of the disease was slightly delayed than in Canezza. The incidence and severity on leaves and fruits were lower in Cirè (Figure 1) than in Canezza (Figure 2). Only the final assessment is detailed here.

![Figure 1](image)

Figure 1. Severity of strawberry powdery mildew on leaves in the different strategies in Cirè
(ANOVA, columns with common letters do not differ from each other according to Duncan test, $P \leq 0.05$)

In both locations biocontrol agents (BCAs), like *Ampelomyces quisqualis*, *Bacillus subtilis* and *Trichoderma harzianum* T39, can control the disease, but to a lesser extent as compared with chemicals, and their level of activity varies among location, environment and time of application. When BCAs were applied in combinations with chemicals a good reduction of residues was achieved while maintaining the same control efficacy against the
disease. Similar results were achieved in the two locations on fruits (Figure 3, only Canezza results are shown).

![Severity of strawberr y powdery mildew on leaves in the different strategies in Canezza (ANOVA, columns with common letters do not differ from each other according to Duncan test, $P \leq 0.05$)](image1)

**Figure 2.** Severity of strawberry powdery mildew on leaves in the different strategies in Canezza (ANOVA, columns with common letters do not differ from each other according to Duncan test, $P \leq 0.05$)

![Incidence of strawberr y powdery mildew on fruits in the different strategies in Canezza (ANOVA, columns with common letters do not differ from each other according to Duncan test, $P \leq 0.05$)](image2)

**Figure 3.** Incidence of strawberry powdery mildew on fruits in the different strategies in Canezza (ANOVA, columns with common letters do not differ from each other according to Duncan test, $P \leq 0.05$)

Chemical residues were found only in strategies 1-3. In particular only azoxystrobin residues were detectable: 0.25-0.30 ppm in strategy no. 1; 0.35-0.4 ppm in strategy no. 2 and 0.15 ppm in strategy no. 3 where azoxystrobin MRL is 2 ppm.

The level of *T. urticae*, and consequently the *A. andersoni* population, was low during all the season thanks to the good ecological balance in the crop in both the locations. The difference between the mites populations in the different strategies was not statistically significant. The BCAs used alone or in the combined strategies did not increase phytophagous mites, neither influence predatory mite populations. Good control of *T. urticae* was also achieved in the chemical strategies.
Discussion

Out of the three described experiments, *T. harzianum* T39 applied alone was significantly lower than the control in three cases, *B. subtilis* in two cases and *A. quisqualis* in one case. In some of these cases the BCAs used alone all over the season did not offer good enough protection of strawberry against powdery mildew. In the combined strategies it is possible to reach a good control and to reduce the number of chemical treatments. In two of the combined strategy it is possible to achieve a low residue (not detectable) on fruits.

All the tested strategies did not increase *Tetranychus urticae*, they had no side effects on *Amblyseius andersoni* populations and consequently they can be regarded as safe for the ecological balance. A further reduction of pesticide application and the optimisation of the integrated strategies will still be possible with a better understanding of the epidemiology of the disease and the identification of the optimal conditions for BCAs application and activity in field.

Acknowledgements

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References

Integrated management of late blight in greenhouse tomatoes

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Late blight, caused by \textit{Phytophthora infestans}, is one of the most devastating diseases of greenhouse grown tomatoes in Israel and elsewhere. For its suppression, growers frequently apply fungicides, but it is not uncommon that severe epidemics develop even in fungicide-treated crops. Determining the quantitative effects of the relevant factors on the pathogen may lead not only to better disease suppression, but also to reduction in fungicide use. The effects of various management actions (covering the soil with plastic, application of fungicides and sanitation), and their interactions, were studied in a series of experiments conducted in walk-in tunnels and commercial-like polyethylene greenhouses. Under conditions of the western Negev (south west Israel) it was found that foliar infection by the pathogen could be suppressed by covering the soil with reflective polyethylene (that resulted in reduction of relative humidity and leaf wetness duration) and by application of fungicides. Under conditions of high temperatures (>20\textdegree C) and dry foliage the infections do not occur. Nevertheless, the pathogen progresses from infected leaf-blades via the petioles, to the stems, where it causes stem lesions. Stem lesions eventually lead to plant death. Observations made in the greenhouses suggested that the damage resulting from stem infection is more significant than that resulted from foliar infection. It is possible to prevent stem infections by sanitation, i.e. removal of infected plant material. Moreover, it was observed that the rate of disease progression in infected leaves was reduced, and fewer plants died from stem lesions, when temperatures exceeded 30\textdegree C. Accordingly, avoidance from opening the side-opening of the greenhouse during the day (which resulted in increased daily temperature) enabled to further improve disease management. In conclusion, the combination of cultural means and spraying control agents at various stages during the growth season results in significant reduction of tomato late blight in greenhouses.
Development of a management system for integrated and biological control of *Botrytis* spp. in flower bulb crops

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*Botrytis elliptica* and *Botrytis tulipae* are the causal agents of leaf blight in respectively lily and tulip (also called ‘Fire’). These *Botrytis* spp. are responsible for losses in lily and tulip production up to 80% (dependent on cultivar). To control *Botrytis* a significant amount of fungicides (25-40 kg/hectare) is used during the growing season. Currently the use of fungicides is under discussion due to environmental concerns and development of fungicide resistance. Moreover, in biological production of lilies and tulips botrytis leaf blight is one of the big problems. At PPO-Flowerbulbs a management system is developed in which several measures are combined resulting in control of leaf blight with no or as less fungicides as possible. Basis for this management system is a *Botrytis* warning system. Through forecasting infection periods it reduces the number of fungicide applications and improves spray efficacy and therefore *Botrytis* control. In such a system other control methods than fungicides can be used also to further reduce fungicide input. Different antagonistic micro-organisms (e.g. yeasts, *Pseudomonas* bacteria) and plant extracts, essential oils etc. are being tested for their *Botrytis* controlling abilities and some have good potential. Furthermore early detection of *Botrytis* in the field may improve the warning system. In addition, other ways to reduce *Botrytis* infection like less dense planting, N-fertilisation (under investigation), crop residue management will be implied into the management system in order to achieve good *Botrytis* control with no or less fungicides.
Entomopathogens and control of insect pests
Use of chemical elicitors to reduce insect pest populations on greenhouse tomatoes

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Abstract: Recent advances in the field of chemical ecology have identified many chemical elicitors that trigger induced resistance and systemic acquired resistance in a variety of plants, including many crop species. Exogenous applications of elicitors have been demonstrated to cause expression of a variety of protease inhibitors or elevated levels of certain plant oxidative enzymes, which in turn are correlated with reduced pests populations on "induced" plants. We report the results of preliminary experiments to assess the feasibility of using applications of four elicitors, Actigard®, Messenger®, ethephon (2-chloroethylphosphonic acid), and methyl jasmonate (MJ) to reduce populations of insect pests commonly infesting commercially grown greenhouse tomatoes in Pennsylvania. Application of ethephon and MJ caused significant elevations in the levels of peroxidase and polyphenol oxidase in tomato leaf tissue. In studies of tomato plants infested with green peach aphids, Myzus persicae, applications of the elicitors Actigard and MJ significantly depressed the rate of aphid population growth. Further studies are underway to optimize the timing of elicitor applications and to determine if other greenhouse pests or diseases are affected by elicitor applications. Biological control failures are frequently attributed to the inability of insect pathogens and natural enemies to keep up with rapid development and reproduction of the target pest. Slowed pest development and reproduction following application of elicitors to crops, may offer improved opportunities for successful biological control.

Key words: chemical elicitors, tomatoes, induced defenses, insect pests

Introduction

Plants respond to environmental stress in a variety of ways, which often enable them to better tolerate these stresses (Ayres, 1992; Karban & Baldwin, 1997). In response to herbivory many plants exhibit Induced Resistance (IR). Resulting changes in secondary chemicals, oxidative enzymes and cuticular defensive structures may enable plants to better tolerate herbivore feeding and reduce herbivore performance. In response to plant pathogens, plants may exhibit Systemic Acquired Resistance (SAR) which may help to reduce the effects of pathogen infection. Improved understanding of plant signaling pathways has opened the possibility of making exogenous applications of chemical elicitors to trigger IR and SAR in crop plants as a means of protecting them against arthropod pests and plant pathogens (Stout et al., 2002). We report the results of experiments in which four chemical elicitors, Actigard, Messenger, ethephon and methyl jasmonate (MJ), were applied to tomato plants to determine if this strategy can be used to manage greenhouse insect pests. Actigard, active ingredient Benzothiadiazole, is a functional mimic of salicylic acid (SA), and activates the SA dependent signaling pathway associated with SAR. Messenger contains the bacterial protein Harpin, derived from Erwinia amylovora, and is reported to activate the SA dependent and ethylene/jasmonic acid dependent pathways, leading to SAR and IR. Ethephon is metabolized within the plant and activates the ethylene dependent pathway leading to SAR and IR. MJ
activates the jasmonic acid dependent pathway leading to IR.

Materials and methods

Plants
Tomato plants *Lycopersicon esculentum*, (cv. Trust) were grown hydroponically using commercial tomato fertilizer and sterile growth media (peat, perlite, vermiculite, 55:20:25).

Target pests
Green peach aphids, *Myzus persicae* (*Homoptera: Aphididae*) were maintained on tomato plants (cv. Trust). Western flower thrips, *Frankliniella occidentalis* (*Thysanoptera: Thripidae*) were maintained on turnip plants (cv. Purple Top Globe).

Elicitors
In initial experiments high, medium and low doses of elicitors were used. For Actigard and Messenger, high, medium and low doses corresponded to twice label, label and half label rates respectively. Ethephon was obtained as 2-chloroethylphosphonic acid (Sigma Chemical Company, St. Louis, MO, USA) and applied as high, medium and low doses of 10 mM, 5 mM and 1 mM solutions respectively. MJ (Bedoukian Research, Danbury, CT, USA) was applied as high, medium and low doses of 10 mM, 7.5 mM and 5 mM solutions respectively. Actigard, Messenger, and ethephon were dissolved in water. Preparations of MJ were first dissolved in 2 ml of ethanol and then added to appropriate volumes of water.

Effects of elicitors on plant growth
Elicitors were applied at high, medium and low doses to young four-leaf tomato seedlings according to a randomized complete block design, to look for evidence of phytotoxicity and other impacts on plant growth. Plant heights were measured 21 days after treatment.

Effects of elicitors on plant oxidative enzymes
Young leaves from elicitor-treated plants were harvested at 72 hours after treatment and processed according to standard methods (Thaler et al., 1996), to look for elicitor effects on the oxidative enzymes, peroxidase and polyphenol oxidase.

Effects of elicitors on aphid performance
Studies looking at elicitor effects on aphid performance used label rates of Actigard and Messenger, and solutions of MJ and ethephon, which were 7.5 mM and 4 mM, respectively. Elicitors were applied to four leaf tomato plants, which had previously been infested with 10 *M. persicae* nymphs. Plastic bags were used during the application process so that neither aphids nor the leaflet on which they were located were contacted by the elicitor treatments. Growth of aphid populations was subsequently monitored for 17 days.

Results and discussion

Effects of elicitors on plant growth
Ethephon had severe impacts on plant growth, but plants treated with the lowest dose partially recovered. MJ treatments caused low levels of leaf spotting and slight yellowing of plants, but did not influence the ability of plants to subsequently flower and produce fruit. Elicitor treatment had a significant effect on plant height at 21 days post treatment (ANOVA F=128.9; df=13,112; \( P \leq 0.001 \)). High and medium doses of ethephon caused permanent stunting (Figure 1). MJ treatments and the lowest ethephon dose caused slight retardation of plant growth. Actigard and Messenger had no detectable impacts on plant growth.
Figure 1. Effect of elicitors on mean plant height measured 21 days after treatment. Means based on nine plants per treatment. Columns with different letters are significantly different by Tukey's test ($P \leq 0.05$).

**Effects of elicitors on plant oxidative enzymes**
Elicitor treatments had significant effects on leaf peroxidase (ANOVA $F=10.0; \text{df}=13,112; P<0.001$) and leaf polyphenol oxidase (ANOVA $F=37.8; \text{df}=13,112; P<0.001$) activity. Medium and high doses of ethephon and MJ caused significant increases in peroxidase activity (Figure 2 upper), while all doses of MJ caused significant elevations in polyphenol oxidase activity (Figure 2 lower).

**Effects of elicitors on aphid performance**
Applications of the elicitors Actigard and MJ significantly depressed the rate of aphid population growth (Figure 3). The fact that aphid population growth was inhibited on plants treated with Actigard, while being stimulated on plants treated with ethephon, strongly suggests that oxidative enzymes are not responsible for reduced growth rates of aphid populations. Given that elicitors were not applied directly to the leaves on which aphids were located, it seems likely that the resulting reductions in aphid population growth rates on plants treated with Actigard or MJ was due to some systemic response within the plant. This may have been due to the induction of some toxic or anti-nutritive agent carried in the phloem or alternatively due to some change in the nutritional composition of materials moving through the phloem. Regardless of the specific underlying mechanisms responsible, applications of Actigard and MJ did substantially decrease the rate of aphid population growth. If defenses induced in tomato following application of elicitors leads to reduced aphid fecundity or a reduced rate of development, this may have important implications for the feasibility of establishing aphid population control by natural enemies or insect pathogens. Reductions in fecundity or increases in development time would facilitate the establishment of population regulation by natural enemies. Meanwhile increases in development time, through lengthening of the nymphal instars, would increase the time available for infective fungal propagules to penetrate the aphid cuticle prior to shedding of the old cuticle at the next molt. Both of these scenarios could significantly improve the likelihood of successful aphid population control within greenhouse settings.
Figure 2. Effect of elicitors on activities of (upper) peroxidase and (lower) polyphenol oxidase in tomato leaf tissue. Enzyme activities calculated as change in optical density (mAbs units) of substrate, per minute, per gram of leaf material. Mean enzyme activities calculated from independent enzyme assays of single leaflets taken from nine plants. Bars show standard errors. Columns with different letters are significantly different by Tukey’s test \((P \leq 0.05)\).

Figure 3. Effect of elicitor treatment of tomato plants on subsequent growth of aphid populations. Mean aphid population sizes based on averages from three cages, each cage average based on three individual plants. Bars show standard errors. * Means for Actigard and MJ significantly lower than controls \((P \leq 0.01)\) at 13 and 17 days. Results representative of a second independent trial.
Acknowledgements

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References

Protoplast fusion, using nitrate non-utilizing (nit) mutants in the entomopathogenic fungus *Verticillium lecanii* (*Lecanicillium* spp.)

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**Abstract:** Commercial biocontrol agents Mycotal and Vertalec have high virulences against whitefly and aphid, respectively. Strain B-2, which was isolated from green peach aphid in Japan, had a high epiphytic ability on plant leaf surface, whereas Mycotal and Vertalec did not. Protoplast fusion was then performed among three strains of *Verticillium lecanii* to obtain useful new strains that had both high virulence and colonization ability. The nit mutants were used to visually select protoplasts that had undergone fusion. The fusion experiment was attempted by combining nit mutants of complementary phenotype. Then 126, 44 and 4 fusion derived colony isolates were detected using combinations of Vertalec×Mycotal, B-2×Mycotal, and B-2×Vertalec, respectively. Furthermore the phenotype of these fusion derived colony isolates was evaluated according to the colony morphology, as a consequence of this morphological characteristics were various compared to original isolates. This paper presents further genomic and pathogenicity analysis with regard to these fusion derived colony isolates.

**Key words:** protoplast fusion, nit mutant, *Verticillium lecanii*

**Introduction**

*Verticillium lecanii* is an entomopathogenic fungus, which has an extremely wide host range (Hall, 1981, 1982, 1984); some strains were used as biocontrol agents because of their high virulence and epizootic efficiency. For example, Vertalec and Mycotal have both been exploited as commercial biological control agents against the greenhouse aphid and whitefly, respectively (Hall, 1984). Strain B-2, isolated from green peach aphid at Obihiro, was found recently to have high colonization ability on living plant surfaces under low nutrients and low humidity conditions.

The nit mutants were commonly used to determine VCGs by complementation testing in several fungal species (Joaquim & Rowe, 1990, Sugimoto et al., 2002, Sugimoto et al., 2003). The major advantage of using nit mutants is their ability of easy selection: unintended mutation does not occur without mutagen treatment. Genetic investigations of parasexual cycle have been performed. Nevertheless, there was the hedge of vegetative compatibility when we bred new strains for biological control, at least in *V. lecanii*. It was overcome using protoplast fusion (Jackson & Heale, 1987). This study performed fusion experiments using Vertalec, Mycotal, and B-2, using nit mutant as genetic marker. This is the first study to use nitrate non-utilizing mutants for visualizing markers of protoplast fusion.
Materials and methods

Fungal strains and generation of nitrate non-utilizing (nit) mutants
This study used three strains of *V. lecanii*. Vertalec and Mycotal are commercial preparations from Koppert UK Ltd. (Wadhurst, East Sussex, UK) and strain B-2, which has high colonizing ability on the leaf surface. This study used water agar chlorate medium (WAC), containing in 1 l of distilled water 2% agar, 0.02% glucose, and 2% KClO₃ to generate nit mutants (Korolev & Katan, 1997)

Protoplast formation and fusion experiments
Conidia spore of parental nit mutants for protoplast production were treated in 5 ml of enzyme solution [(Novozyme 188 (0.01 g/ml) and Lysing Enzyme (0.01 g/ml) in 1 M MgSO₄)]. Protoplasts were centrifuged at 2000 g for 5 min. These pellets were resuspended in sorbitol solution (1 M sorbitol, 50 mM CaCl₂, 10 mM Tris-HCl pH 7.4), then further adjusted to ca. 1 × 10⁶ conidia/ml. Each parental protoplast suspension of complementary combination was mixed, treated with prewarmed (30°C) 30% PEG 4000 (10 mM CaCl₂, 10 mM Tris-HCl pH 7.5) and incubated at 30°C for 15 min (Anne & Peberdy, 1975). This mixture was centrifuged at 1000 g for 5 min and resuspended in sorbitol solution, then plated on minimal medium (MM). When wild-type growth colonies were detected, these were considered to be fusion-derived colonies. To purify them, each of these colonies was translated on MM again; only mycelia that showed prototrophic growth were transferred to PSCA as fusion derived colony (FDC) isolates.

Morphology of fusion derived colony isolates
Mycelial plugs of FDC isolates and parental nit mutants were inoculated on centers of plates (90 mm diameter) containing PSCA, then incubated as described above for about 2 week. Colony morphology was distributed visually by color, figure, the quantity of aerial mycelium, and presence of sector.

Results and discussion

Generation and characterization of nit mutants
The wild-type colony of two isolates became very restricted on WAC; chlorate resistant sectors readily appeared as thin, fast growing, and fan like sectors 16 d after inoculation. 31.1% and 36.1% of recovered chlorate resistant sectors showed thin expansive colonies with no aerial mycelium when subcultured on MM. These sectors represented nit mutants (Table 1). The obtained nit mutants were determined to be four phenotypes (nit1, nit2, nit3, nitM) depending on five media containing different nitrogen sources: nit1 and nitM were detected, but nit2 and nit3 were not apparent. The proportions of nit1 and nitM from Vertalec were 42.9% and 57.1%, whereas those from Mycotal were 30.8% and 69.2% (Table 1). These nit mutants were named 1-A-F, M-A-H, 1-a-d, and M-a-i, respectively.

In complementary tests to select tester mutants for protoplast fusion, intra-isolate pairings between all combinations of nit1 and nitM were performed in three isolates. Vertalec and Mycotal were self-compatible, but B-2 was self-incompatible. 1-A, M-B and 1-b, M-a, which showed compatibility at high frequency and formed stronger heterokaryons, were selected from nit mutants of Vertalec and Mycotal, respectively. From those nit mutants of B-2 that were indicated as self-incompatible, we selected 5-2, which had vigorous viability, for the fusion experiment.

Protoplast fusion
Kao & Michayluk (1974) reported protoplast fusion with PEG in 1974. Anne & Peberdy (1975) attempted applying plant protoplast fusion technique to filamentous fungus for the first time. Subsequently, their method was followed extensively as a fungal fusion technology. Trans-
formation has been used recently as a method to integrate specific useful genes. Nevertheless, protoplast fusion was suitable in the character that the gene could not be isolated or the character to be ruled by not single gene but polygenes.

Table 1. Frequency and phenotype of nit mutants recovered from two isolates of V. lecanii on WAC medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of Inoculations a</th>
<th>No. of sectors b</th>
<th>No. of nit mutants c</th>
<th>nit (%) mutants</th>
<th>Phenotype of nit mutants %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertalec</td>
<td>21</td>
<td>45</td>
<td>14</td>
<td>31.1</td>
<td>42.9 57.1</td>
</tr>
<tr>
<td>Mycotal</td>
<td>12</td>
<td>36</td>
<td>13</td>
<td>36.1</td>
<td>30.8 69.2</td>
</tr>
</tbody>
</table>

aNumber cultured on WAC.
bNumber recovered on WAC.
cNumber of chlorate resistance colony that grew as thin expansive colonies on WAC.

After fusion treatment, primary fusion derived protoplast suspensions were visually selected on MM plates. Wild-type growth colonies were found among thin, expansive mycelium. To purify and select for stable fusion derived colonies, these prototrophic colonies were subcultured individually to fresh MM plates. Colonies with sectors were transferred singly. Only the inocula that indicated wild-type growth on this plate were considered stable FDC isolates. Therefore 44, 4, 20, and 106 of stable FDC isolates were obtained from 5-2 × M-a, 5-2 × M-B, 1-b × M-B, and 1-A × M-a. These isolates were named 2aF1-44, 2BF1-4, BbF1-20, and AaF1-106, respectively. So far, fusion experiments of combination 5-2 × M-B obtained only four isolates. Zare & Gams (2001) suggested that V. lecanii was accommodated in the genus Lecanicillium and reclassified in several species, based on morphological observations and molecular analysis. Vertalec was reassorted in L. longisporum; Mycotal and B-2 belonged to L. muscarium. From this viewpoint, one may say that fusion experiments of Vertalec × Mycotal and Vertalec × B-2 showed intraspecific or interspecific hybridization. Bos (1996) reported that protoplast fusion between interspecific or unrelated strain was difficult. One explanation for the lack of successful fusion of 5-2 × M-B may be that this was an interspecies combination.

**Morphological evaluation**

Morphologies of nit1 and nitM from the same parental nit mutant were similar: nit mutants from Vertalec showed a thin colony with short mycelium; those from Mycotal showed dense abundant aerial mycelium; and those from B-2 showed coarser long mycelium. However, morphologies of FDC isolates were considerably diverse. All these colonies were distributed roughly into seven classes: those resembling parental nit1 or nitM, parental nit mutant partially mixed with the other, thick, double circle, radial growth, and atypical growth. Atypical colonies showed various patterns: colonies with strong yellow, or sectors, or without rondure. These results of various morphologies of FDC isolates were inferred to result from changes of nuclear phase after protoplast fusion, and occurring recombination side by side.

The nit mutant was a useful fusion marker in V. lecanii. Furthermore, genomic rearrangement or genetic recombination can be expected to occur because morphology varied greatly as colonies appeared. Therefore, protoplast fusion techniques have the potential of improving virulence or epizootic efficiency of Deuteromycete entomopathogenic fungi, exactly as we intended. After molecular analysis, investigation based on virulence tests and evaluation of colonization ability will be performed according to these genetic exchanges whether or not virulence or colonization ability coexists. Ultimately, we will select a useful new strain as a biological control agent.
Acknowledgments

The authors thank Koppert UK Ltd. for permission to use Vertalec and Mycotal samples.

Reference

Experiences with the entomopathogenic fungus *Beauveria brongniartii* for the biological control of the common cockchafer *Melolontha melolontha*

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Melocont®-Pilzgerste, a commercial product based on barley kernels colonised by *Beauveria brongniartii*, was tested against the common cockchafer *Melolontha melolontha* in large field trials over a period of eight years. The barley kernel product was applied in pastures with a slit seeder at various times of the year. The highest efficacy of the product was achieved by incorporating the inoculum into the soil at a depth of 3 to 10 cm. A threshold of inoculum in soil was required to ensure epidemic levels in the pastures (>2 x 10⁴ spores g⁻¹ dry weight of soil). The results of field trials conducted between 1995 and 2003 with the barley kernel product indicated that the density of *B. brongniartii* increased continuously after each of the applications as long as the target pest was present. With the exception of a site with sandy-textured soil, fungal populations within soils persisted above the threshold level. Re-isolation studies showed a uniform vertical distribution of fungal spores at depths of 10 to 20 and 20 to 30 cm, respectively.

Microsatellite marker analysis confirmed that the applied strain and re-isolated strains were identical. The application of the *B. brongniartii* barley kernel product resulted in a sufficient suppression of cockchafer populations after only 2 years of applications (>20 % prevalence of mycosis), and populations were reduced from > 70 larvae / m² to less than 22 larvae/m² after 5 years. After that, no relevant damages by *M. melolontha* have been reported at treated sites. The described biocontrol agent was registered by the Austrian plant protectant legislation as of June 2000 based on the results of this study.

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Antagonistic activity of the entomopathogenous fungus
*Beauveria bassiana* against grapevine pathogens:
perspective of combined use against insects and fungi

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Abstract: Pathogenic activity against several species of insects of some fungi belonging to the *Beauveria* genus is well known and they can be used in organic and integrated pest management of several crops. A partial antagonistic activity has been reported also against some fungi and bacteria. A combined activity against insects and fungi could be an additional tool for biological pest control. The inhibitory activity *in vitro* of a wild strain of *B. bassiana* (B1) isolated from dead insects, has been evaluated against two plant pathogens: *Armillaria mellea* and *Penicillium digitatum*. Dual culture test with the isolate and the pathogens has been used. In this test pathogen and biocontrol agent growth at different temperatures has been evaluated with the software Image Pro Plus. In the first stage the isolate seems to have antibiotic activity against the two pathogens and in a later stage B1 shows a partial hyperparasitism against *A. mellea*. The inhibition of *P. digitatum* is lower than *A. mellea* and the pathogen also shows a light antagonistic activity against B1. The effect against mites has been evaluated.

Key words: *Armillaria mellea, Beauveria bassiana, Penicillium digitatum*, antagonistic activity, mite, side effect

Introduction

*Beauveria bassiana* is a common soilborne fungus that occurs worldwide (Benuzzi et al., 2001). It attacks a wide range of both immature and adult insects and mites (Saenz de Cabezon et al., 2001). There is a high number of specific strains, which have considerable variation in virulence, pathogenicity and host range. Insect death may result from a combination of actions, including depletion of nutrients, physical obstruction or invasion of organs and toxicosis. *Beauveria bassiana* produces a number of toxic compounds, including beauvericina, bassianolide and oosporein (Douglas et al., 2003; Strasser et al., 2000). After the insect death, the fungus often grows saprophitically within the host, and the produced metabolites may be involved in a competitive exclusion of competing micro-organisms, like plant pathogens and Gram+ bacteria (Strasser & Abenstaind, 2000; Vey et al., 2001).

The aim of this work was to test the potential biocontrol activity against plant pathogens of a new isolate of *B. bassiana* (B1). Since it is often assumed that *B. bassiana* strains are safe for beneficial mites (*Phytoseids*) and they do not influence the parasite *Tetranychus urticae* Koch, we aimed also to evaluate B1 side effects on mites.
Material and methods

A strain of *Beauveria bassiana* (named B1), isolated from the insect *Corithuca ciliata*, was used in vitro tests against two plant pathogens: *Armillaria mellea*, which causes root rot in many woody and herbaceous plant worldwide and *Penicillium digitatum*, which is a post harvest fruit pathogen.

**Antagonistic activity evaluation**

The antagonistic activity of B1 was in vitro evaluated. Plant pathogens and B1 were inoculated in the same Petri dish, at a distance of 2 cm. Different medium and conditions were used and reported in Table 1. The growth of the two pathogens (with and without the isolate B1) was evaluated at several times after inoculation with the Image pro Plus software until 30 days after inoculum.

Table 1. Experimental conditions

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Medium</th>
<th>Temperature</th>
<th>Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Armillaria mellea</em></td>
<td>Malt extract agar</td>
<td>5, 15 and 25°C</td>
<td>10 days before B1</td>
</tr>
<tr>
<td><em>Penicillium digitatum</em></td>
<td>Potato dextrose agar</td>
<td>20°C</td>
<td>At the same time of B1</td>
</tr>
</tbody>
</table>

**Side effect on mites**

The side effects on mites (*Tetranychus urticae* and *Amblyseius andersoni*) were estimated by measuring the direct toxicity on adult females and effect on reproduction. Pairs of non-coeval females were placed on leaf disks and three replicates were considered. Six females on a leaf disk were treated with a Potter spray tower with 8 ml of 5x10^6 conidia/ml water solution. Distilled water and reference product (Pyrethrum) was also tested. Mortality and female fecundity were assessed for ten days.

Results and discussion

The *A. mellea* area measures, with and without B1, at 25, 15 and 5°C are presented in Figures 1, 3 and 4, *P. digitatum* growth, with and without B1, at 20°C is shown in Figure 2. B1 strain has good in vitro antagonistic activity against both plant pathogens at higher temperatures (Figures 1 and 2). Growth of both *A. mellea* and B1 is slowed down at lower temperatures. Antagonistic activity is visible only after a growth of 100 mm², e.g. at the end of the test time at 15°C (Figure 3), and is not reached at 5°C (Figure 4). After 30 days, B1 shows a partial hyperparasitism against *A. mellea* and *P. digitatum* shows also a slight inhibition activity against B1 (results not shown).

The effect of the B1 on the *A. andersoni* strain were similar to water control, in contrast the references Pyrethrum showed a high toxicity level. Neither did B1 interfere with the populations of *T. urticae*. These preliminary results show that *B. bassiana* B1 is a promising strain for biocontrol of pathogens and could be implemented for the combined control of insect and plant pathogens.
Figure 1. Growth of *Armillaria mellea* with and without B1 at 5°C

Figure 2. Growth of *Armillaria mellea* with and without B1 at 15°C

Figure 3. Growth of *Armillaria mellea* with and without B1 at 25°C

Figure 4. Growth of *Penicillium digitatum* with and without B1 at 20°C
Acknowledgements

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References

Selection of entomopathogenic nematodes for heat tolerant and desiccation traits

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Entomopathogenic nematodes (EPNs) are currently used in biological control of insect pests, but their sensitivity to environmental constraints reduces their application possibilities. Thus there is the need to find natural EPN strains that are able to survival to environmental stress. In this study we present the selection for heat tolerance under laboratory conditions of Dauer Juveniles (DJs) larvae of five EPN strains. Two of the five strains showed heat tolerance ability since more then 75% of their DJs survived after 30 minutes exposure at 40°C. The two selected strains were also tested for their ability to survive to low humidity conditions. Surviving was assessed after exposure of nematodes to different relative humidity (RH) levels in sealed desiccators. DJs were able to survive several hours in absence of water in liquid state in a chamber with 100% relative humidity. Additionally, the two strains were able to survive at 85% RH treatment for two hours. The advantages of using heat and desiccant tolerant entomopathogenic nematodes in biological control program are discussed.
Development of a bio-insecticide based on a cold-active entomopathogenic nematode

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The larvae of *Otiorhynchus sulcatus* F. (*Coleoptera, Curculionidae*) feed on the root system of several plant species (mainly ornamentals and nursery stock). Biological control is a promising alternative approach to the chemical strategy, which has been accused to have a negative impact on the environment and on the insect resistance. It is very well known that entomopathogenic nematodes can effectively control the larvae of this pest, however the commercially available strains are only effective at temperatures above 12°C. On the other hand, the larvae of *O. sulcatus* develop also during winter and they feed even at temperatures as low as 5°C; for this reason nematodes active at these low temperatures could be very useful for the biocontrol of these insects.

Several nematode isolates found in soil collected in 1994 in the Italian Alps were screened under conditions as close as possible to the real environment in which the insect develops. Potted plants grown on peat, infected with larvae of *O. sulcatus* and treated with $0.5 \times 10^6$ nematodes/m² were used and results were evaluated between 14 to 21 days after treatment. The first screening has been done at 12°C, then 8°C and at the end, 5°C. At this last extreme temperature, only one of those isolates gave insect mortality between 80 to 90%. A commercial strain of *Steinernema feltiae* was always used as the reference material. The efficacy of this commercial strain was comparable to the cold active isolate described here, only when tested at 20°C, and it was far lower at extreme temperatures. The described isolate can be used for the control of *O. sulcatus* larvae within a wide range of temperatures from as low as 5°C to a maximum of 20°C. In field trials, the selected nematode demonstrated to be very effective at very low temperatures. By mean of RAPD-PCR analysis, it has been established that the nematode belongs to the species *Steinernema kraussei*. In an *in vivo* production test, the cold-active isolate showed an optimal growing temperature of 15°C. In liquid fermentation satisfactory yields were obtained also at 20°C.
Combined use of insect pathogenic fungi and nematodes against the onion thrips, *Thrips tabaci*, in the field

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**Abstract**: In the year 2003, field trials against *Thrips tabaci*, an important pest insect in onion, leek and chives, were conducted at four different sites with commercially available products based on insect pathogenic fungi and nematodes. The trials were performed according to the EPPO guideline PP 1/85(3) for the efficacy evaluation of insecticides “Thrips on Outdoor Crops”. As a chemical standard Perfekthion® was used in onion. In leek, Spruzit® and Neudosan® were applied alternately. The biocontrol products used were Mycotal® (*Lecanicillium muscarium*, former *Verticillium lecanii*, Koppert NL), PreFeRal® (*Paecilomyces fumosoroseus*, Biobest BE), Naturalis L® (*Beauveria bassiana*, Intrachem IT), Nemaplus® (*Steinernema feltiae*, E-Nema DE) and Nemagreen® (*Heterorhabditis bacteriophora*, E-Nema DE). They were applied either alone or in a mixture using common spray equipment. A wetting agent was added. The nematodes were applied at a rate of 1x10^6 infective juveniles/m². The dosage of the fungi was 1 kg (1.5 l in the case of Naturalis L®)/ha. The treatments started mid June and were repeated up to six times in weekly intervals. Throughout the summer, *T. tabaci* was recorded only in medium numbers (mean of 30 specimen/plant max.) at all four sites. In two trials (onion, Mainz; chives, Horkheim), no differences between treatments and the untreated control could be detected. In the third trial (onion, Schifferstadt), a significant reduction was recorded for the treatment ‘PreFeRal+Nemaplus’, both in the number of thrips/plant (2 compared to 6 in the control, respectively) and the frequency of infestation (38% compared to 93% in the control, respectively), one week after the final application (three times in total). Also in the fourth trial (leek, Bonn), the number of thrips/plant was lowest for the treatments ‘fungus+nematode’ (4 compared to 7 in the control, respectively; fungus here: Mycotal®) one week after the final application (six times in total). In this trial, yield was measured additionally, and it turned out, that the weight/plant registered for the treatments ‘Nemaplus’ and ‘Mycotal+Nemaplus’ was 20% higher than in the control (425 and 412 g/plant compared to 345 g respectively). These trials show that the use of insect pathogenic fungi and nematodes in the field is possible, confirming results of others collected in Great Britain and The Netherlands. In view of the extreme weather conditions in summer 2003 in Germany, and in comparison to the effect of the chemical standards, the results gained with the biocontrol agents are regarded as very promising. However, more experiments are necessary.

**Keywords**: *Thrips tabaci*, insect pathogenic fungi, *Beauveria bassiana*, *Lecanicillium muscarium* *Paecilomyces fumosoroseus*, insect pathogenic nematodes, *Heterorhabditis bacteriophora*, *Steinernema feltiae*, biocontrol, field testing

**Introduction**

Thrips can cause serious damage to many different crops. Especially the onion thrips, *Thrips tabaci*, is an ubiquitous pest insect. Due to their hidden mode of life, thrips are difficult to control. In particular, organic growers have no effective and economic control measure available against thrips.

The present study was conducted within the frame of the "Bundesprogramm Ökologischer Landbau“, a German research programme designed for support of organic agriculture. The intention was to develop a sustainable control method against both the air- and the soilborne life stages by combining insect pathogenic fungi with nematodes. After
laboratory and greenhouse testing, field experiments were performed in 2003. The results are reported here.

**Material and methods**

In summer 2003, field trials were conducted in different crops and at four different sites: Bonn – leek (variety ‘Helios’), Mainz and Schifferstadt – onion (variety ‘Bristol’), Horkheim – chives, in co-operation with plant protection services and research institutions. As far as possible, all trials were performed according to the EPPO Guideline for the efficacy evaluation of insecticides PP 1/85(3) “Thrips on outdoor crops“. Briefly: Plot size at least 20 m²; four replicates per treatment; untreated control and a chemical reference product included; assessments by counting the number of living thrips either at five random groups of four successive plants per plot (leek) or on the plants of 5 x 0.5 m row lengths per plot (onion, chives). Perfekthion® served as chemical reference in the onions, whereas in the leek experiment, Spruzit® and Neudosan® were applied in alternation. As biological treatments the following products were used: Mycotal® (Lecanicillium muscarium, formerly: Verticillium lecanii, Koppert NL), PreFeRal® (Paecilomyces fumosoroseus, Biobest BE), Naturalis L® (Beauveria bassiana, Intrachem IT), Nemaplus® (Steinernema feltiae, E-Nema DE) and Nemagreen® (Heterorhabditis bacteriophora, E-Nema DE). They were applied either singly or in a mixture by means of standard application equipment (motorized sprayer, nozzle type e.g. Teejet 8003 VK, 2-3 bar), addition of a wetting agent (either Addit® or ProFital® fluid) and – for the nematodes only – removal of all filters. The application rate was 1x106 infective juveniles/m² for the nematodes, and 1 kg (respectively 1.5 l for Naturalis L®)/ha for the fungi. All treatments were applied with 1000 l of water/ha. Starting from mid/end of June, the plants were treated in weekly intervals up to six times. In addition, at the field site in Schifferstadt a fungicide commonly used in practice was applied against downy mildew, but with a distance of three days to the biological treatments.

**Results and conclusions**

At all sites, only medium infestation rates were recorded throughout the summer (mean of 30 thrips/plant at maximum). In two trials (onion – Mainz; chives – Horkheim) no differences between the treatments and the untreated control could be detected at the end. In the onions, the frequency of infestation was 70-78% for all variants one week after the last treatment (five times in total), but the number of thrips was very low (2-3 insects/plant). Here, many natural enemies of thrips were observed (e.g. Aiolothrips spp., Chrysoperla-larvae) during the season. In the chives, a degree of weed covering of ≥70% at the date of the final assessment prevented an appropriate evaluation. In contrast, in a third trial (onion – Schifferstadt) a significant reduction of both, the number of thrips/plant and the frequency of infestation was achieved one week after the last treatment (3 times in total) for the variant ‘PreFeRal+Nemaplus’ (ca. 60% effect compared to ca. 20% for the chemical reference, Perfekthion®, respectively, see Figure 1). Also in the fourth trial (leek – Bonn), the lowest number of thrips/plant (4 compared to 7 in the control, respectively) was recorded within the ‘fungus+nematode’-variants (fungus here: Mycotal®) one week after the last treatment (6 times in total). No differences between variants were recognized for the frequency of infestation. In this trial, the yield was assessed additionally. Interestingly, the weight/plant was approx. 20% higher for the variants ‘Nemaplus®’ and ‘Mycotal® +Nemaplus®’ in comparison to the control (425, 412 and 345 g/plant, respectively).
Figure 1. Effect [%] of treatments 2-8 in the field trial in Schifferstadt [Perfekthion® (2), PreFeRal® (3), Nemagreen® (4), Nemaplus® (5), Mycotal® (6), PreFeRal® + Nemagreen® (7), PreFeRal®+Nemaplus® (8), respectively] in comparison to the control (6.5 thrips/plant and 93 % frequency of infestation). Different letters indicate significant differences (Duncan, p≤0.05)

These trials confirm previous results from Great Britain and The Netherlands, which also showed that the use of insect pathogenic nematodes and fungi is not necessarily restricted to protected crops and greenhouses, but can be extended to the open field. In view of the extreme weather conditions in summer 2003 (very hot and dry) and in comparison to the effect of the chemical references, the results obtained with the biocontrol agents are encouraging. Unfortunately, the study was not prolonged.

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The use of *Metarhizium anisopliae* against grape phylloxera

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**Abstract**: Bioassay with *Daktulosphaira vitifoliae* (Fitch) showed that they are susceptible against *Metarhizium anisopliae*. First infected insects could be found five days after treatment. In pot experiments, using *Metarhizium* colonised barley as biocontrol agent (BCA), only nodosities without any living phylloxera could be found in eight of ten treated plants after thirty-two days. The control group was still heavily infested with grape phylloxera of all instars. A field study was initiated in 2003. The treatment with *M. anisopliae* colonised barley led to a decrease of phylloxera infestations. No negative effects on non-target invertebrates (*Acari, Collembola, Carabidae, Lumbricidae*) or on soil fungi could be observed.

**Key words**: biocontrol, entomopathogen, Hyphomycetes, *Metarhizium anisopliae*, grape phylloxera, *Daktulosphaira vitifoliae*

**Introduction**

Grape phylloxera, *Daktulosphaira vitifoliae* (Fitch), is a serious pest of commercial grape-vines worldwide. On fresh roots, the grape phylloxera causes so called nodosities, beak-like swellings, as a result of feeding activity. High populations of this pest can result in premature defoliation, reduced shoot growth, reduced yield, and reduced quality of the crop, and even crop death. Currently, registered chemical or biological control agents against phylloxera are not available in Europe. The use of systemic insecticides (e.g. Thiamethoxam, and Imidacloprid) is recommended in California (UC Pest Management Guidelines, 2003) but it is very restricted, e.g. to irrigative farming systems and very short entry levels. BCAs are considered as a real alternative but little information is available. English-Loeb et al. (1999) conducted pot trials with entomopathogenic nematodes and found these to be ineffective against phylloxera; Granett et al. (2001) mentioned that the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. controlled phylloxera in vitro, but no data were made available. Recently, *Metarhizium anisopliae* (Metschn.) Sorok. is proposed to act as an effective BCA (Kirchmair et al., 2004; Huber et al., 2004). Therefore, the objective was to estimate the potential of *Metarhizium* as BCA against grape phylloxera, by evaluating plant growth and phylloxera infection of the plants in pot experiments and field studies.

**Material and methods**

**Plant material, grape phylloxera and fungal isolate**

The grape clone 5BB Klon 13-11 Gm was used for pot experiments. The trial site for the field experiment was a commercial vineyard near Geisenheim, Germany planted with “Weißer Riesling” on rootstock 5°C (both rootstocks: *Vitis berlandieri x V. riparia*). The *Metarhizium*
anisopliae var. anisopliae strain Ma 500 was used for the bioassay as well as for pot experiments, strain BIPESCO 5 for the field trial. Both fungal strains were grown on barley kernels according to Strasser (1999).

**Bioassay**
Phylloxera infected grape roots were washed with sterile tap water, placed on moist filter paper in sterile culture plates and sprayed with Ma 500 spore suspension. After 12 h incubation a visual assessment was carried out every 6 hours using a binocular.

**Pot experiment**
Thirty-two grape vine cuttings were planted in microwave-sterilised soil. One grape leaf with approximately 30 phylloxera leaf galls each was incorporated into the soil of sixteen plants. When plants were sufficiently strong infested with grape phylloxera, Ma 500 colonised barley was added. Details on bioassay and pot experiment are given by Kirchmair et al., 2004.

**Field trial**
In spring 2003 a randomised block designed trial was established at a well-kept vineyard near Geisenheim, Germany. Each plot consisted of 16 plants. The vineyard was homogenously infested with grape phylloxera, but showed no obvious damage of the vines (i.e. normal foliation). For applying barley a sowing machine in combination with a rotary harrow was used. With this technique application is possible in the tramline only and not underneath the row. As controls four plots were treated with sterile barley, and four plots remained untreated except for using the harrow.

**Assessment of plant growth and phylloxera infection**
The relative chlorophyll content (RCC) of 10 leaves per plant was measured using a Minolta SPAD-502 Chlorophyll Meter. Grape growth was estimated using modified standards of the O.I.V. (Office international de la vigne et du vin, Paris, France). For the assessment of grape phylloxera populations the method of Porten & Huber (2003) was used.

**Assessment of M. anisopliae density in soil**
From each treatment randomised soil samples were taken with a core borer, mixed, air-dried overnight and sieved through a 4 mm sieve. *M. anisopliae* density was assessed as cfu g⁻¹ dry soil according to Strasser et al. (1996).

**Effects on soil mesofauna and soil microorganisms**
For studying effects on the edaphon soil samples were taken with a core borer (⌀ 50 mm). For a dynamic extraction of the soil fauna the method of Kempson et al. (1963) was applied. Lumbricidae were extracted with a mustard suspension according to Gunn (1992). For the assessment of the impact of fungal colonised barley on soil fungi soil suspensions were plated on potato-dextrose-agar and incubated for one week at 25°C (Längle et al., 2004). The colonies were counted and determined at genus level.

**Results**
The bioassay showed that grape phylloxera is susceptible to *Metarhizium anisopliae* applications. First infected insects could be found five days after treatment. The pot experiment gave further affirmations on the efficacy of *M. anisopliae*. Differences of phylloxera-infestation could be estimated between treated and untreated plants. Thirty-two days after inoculation with *M. anisopliae*, fresh nodosities with few living insects and eggs could be observed only on two plants. This corresponds with the typical class three characteristics of the phylloxera assessment classes. All other samples (i.e. eight out of ten treated plants) were grouped as class one type, showing only old nodosities without any living phylloxera. All of the untreated phylloxera infested plants showed class three to class seven
characteristics. Comparing treated and untreated plants a significantly higher *M. anisopliae* density (7.3 x 10^4 cfu g^-1 dry soil) was estimated in those pots, which were also treated with grape phylloxera (*P*≤0.05).

These results could be reproduced in the field trial. In *Metarhizium* treated plots a reduction of phylloxera infestation could be estimated. No relevant density of *M. anisopliae* could be found in the soil before application. The density in the tramline of treated plots was sufficiently high (10^2-10^6 cfu g^-1 dry soil), while according to the application technique an only low density was detected underneath the row of treated plots (≤10^3 cfu g^-1 dry soil). Two month after treatment the infestation frequency by grape phylloxera was distinctly lower in the tramline of *Metarhizium* treated plots. Also the infestation intensity could be ranked within lower assessment classes in the tramline of treated plots. No difference between control and treated plots could be observed underneath the row, where the *M. anisopliae* density was very low according to the application method.

No differences in the abundances of the orders of soil invertebrates (e.g. *Acari*) or of the abundance of the different species of Collembola and Lumbricidae could be found between the variants (no treatment, sterilised barley, *M. anisopliae* colonised barley). No influence on *Harpalus affinis*, the only observed Carabidae, was estimated. The evaluation of the PDA dilution plates revealed no quantitative or qualitative changes in the composition of cultivable soil fungi.

**Discussion**

Our data suggest that *M. anisopliae* can be used to control *Daktulosphaira vitifoliae*. Although no fungus infected insects or eggs could be observed the efficacy of *M. anisopliae* can be postulated by the following reasons: Previous bioassays with all instars of grape phylloxera showed that *Metarhizium* kills and mummifies the insects (Kirchmair et al., 2004). Corresponding to the size of grape phylloxera (<0.5 mm) a rapid mineralisation in soil is likely. The increase of *M. anisopliae* density in the phylloxera-infested pots and the significant decrease of fresh root galls indicate, that the entomopathogen *M. anisopliae* must have a control effect on grape phylloxera. Moreover, the decrease of phylloxera frequency and intensity in the tramline of treated plots in the field experiment affirms the results of the pot experiments. The estimated *M. anisopliae* density corresponds with published threshold values on entomopathogenic Hyphomycetes, which were applied to control soil dwelling pests in grassland and pastures (Ferron, 1979: >2x10^4 g^-1 dry soil; Strasser, 1999: >10^5 g^-1 dry soil).

*M. anisopliae* has a number of advantages as a biocontrol agent because this fungus is relatively easy to be mass produced, strains can be selected with appropriate levels of virulence and specificity and formulated with increased shelf life and field efficacy (Milner, 2000). The use of a sowing machine in combination with a rotary harrow is a low costs and time-saving application where mechanical damage of the grapes is negligible. But with this method barley can be spread in tramline only and not underneath the row. Future work will be required to estimate if *M. anisopliae* will be spread by different vectors underneath the row also or if alternative method must be applied to bring out colonised barley closer to the rootstock. Moreover, for a broader application, necessary for an area-wide combat of grape phylloxera, methods must be developed to bring out *Metarhizium* products in areas with other cultivation traditions (e.g. cultivation on steep hills).

Although we found no influence on non-target organisms, an important point is to determine the risk for a longer period and on more trial sites with different invertebrate or and microbial cenoses.
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References


Microbial communities
and diversity
Multi trophic relationships – interaction of a biocontrol agent and a pathogen with the indigenous micro-flora on bean leaves

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Abstract: The phyllosphere microflora may interact with the pathogen, the biocontrol agent and the plant and affect the outcome of their interaction. The microflora may be altered when an infected plant is treated by a BCA resulting in a change of the outcome of the interaction to give either better control of the pathogen or enhanced disease levels. We studied bean leaves for multi-level interactions of the BCA Trichoderma harzianum T39, the plant pathogen Botrytis cinerea and indigenous microflora. B. cinerea disease severity on leaves was evaluated following direct T. harzianum treatment to the leaves or root treatment. Culturable microflora on leaf surfaces and inside the leaf tissue were studied following either surface washing or surface leaf disinfection and maceration each followed by conventional plating on growth media. Bacterial 16S ribosomal DNA (rDNA) extraction from bean leaves that were subjected to the various treatments was PCR amplified with the primers 530R and GC-338F. Amplicons were separated by size and base composition by denaturant gradient gel electrophoresis (DGGE) in order to fingerprint shifts in the structure of the natural plant-associated microbial communities (culturable and non-culturabl e) that may result from biocontrol and pathogen activity. B. cinerea-incited disease on bean leaves was significantly reduced when leaves were directly treated with T. harzianum T39 or when a special separation between biocontrol treatment and pathogen leaf infection was kept, i.e. the T. harzianum was applied to the root zone and the pathogen was infected on the leaves. Leaf tissue associated, culturable yeast and bacterial population levels were increased as a result of B. cinerea infection and as a of T. harzianum treatments either to leaf or to roots. Surface phylloplane yeast levels tended to decrease as a result of T. harzianum treatments. Representative isolates from the phyllosphere antagonized Botrytis in vitro and on bean leaves. DGGE performed with the same samples revealed increased variation in the bacterial population during the course of plant incubation as a result of B. cinerea infection. T. harzianum treatment to the leaves or to the root zone resulted in increased variability in the bacterial population inhabiting the leaves. It may be speculated that some of the effect exerted by T. harzianum as a biocontrol agent is associated with microbial changes that occur as a result of is direct application to the leaves or indirect application to the roots. Earlier research revealed other modes of action of T. harzianum i.e. induced resistance, competition for nutrients and space and suppression of B. cinerea pathogenicity enzymes.

Introduction

Research on microbial interactions in agricultural ecosystems has yielded a vast amount of knowledge about biological control of plant pathogens. Infection by pathogens can be reduced by prior inoculation of the plant surfaces with filamentous fungi, bacteria, yeasts or viruses. Biocontrol offers attractive alternatives or supplements to the use of conventional methods for disease control. Microbial biocontrol agents (BCAs) are perceived as being less detrimental to the physical and biological environment and less hazardous than chemical control agents to
human beings. The generally complex mode of action of many biocontrol agents makes it unlikely that resistance will develop in populations of pathogens.

Traditionally biocontrol systems are described with three levels of interaction i.e. antagonist (=BCA) – plant pathogen, pathogen – plant, BCA – plant. However, a forth component has to be considered in this system, namely the indigenous microflora on the plant surface. The microflora may interact with the pathogen, the BCA and the plant and affect the outcome of their interaction. The microflora may be altered when an infected plant is treated by a BCA resulting in a change of the outcome of the interaction to give either better control of the pathogen or enhanced disease levels. In spite of their potential effect on the efficacy of biocontrol, the interaction of BCAs with the host plants, their effect on natural microflora of plant surfaces and the effect of this population on the biocontrol activity in agricultural systems received until now minor or no attention at all.

Among the many potential natural enemies of pathogens, antagonistic fungi have received significant attention, resulting in development of several fungus-based preparations that are already in commercial production and are in use in crop protection. Enthusiasm for the use of biocontrol needs to be moderated by the realization that only a limited number of microbial agents have been registered as commercial products. Among the first products of this kind to be developed and commercialized worldwide is the biocontrol preparation TRICHODEX, that contains isolate T39 of the disease-antagonistic fungus *Trichoderma harzianum*. The proposal deals with the interaction of this antagonist with the various components of the biocontrol system. *T. harzianum* T39 is registered for agricultural use in c. 20 countries including in EU members. It controls several plant pathogens on grape vines, greenhouse crops and orchards (Elad, 2000a; O’Neill, 1996).

*B. cinerea* causes grey mould diseases and other symptoms on many crops, including grapevines, greenhouse vegetables, flowers and ornamentals, and even orchard fruits. It attacks all plant parts including leaves, stems, flowers and fruits in both preharvest and postharvest stages (Elad & Shtienberg, 1995). *B. cinerea* is one of the pathogens that *T. harzianum* T39 controls. Several modes of action are involved in the activity of *T. harzianum* T39, including novel ones that have not been described previously for other BCAs (Elad, 2000b). These mechanisms include: i. involvement of locally and systemically induced resistance that has been demonstrated when application of living cells of T39 to the roots and of dead ones to the leaves of plants resulted in suppression of *B. cinerea* on bean, tomato and pepper leaves, and of powdery mildews on cucumber, pepper and tobacco leaves; ii. T39 suppressed pathogenicity enzymes of *B. cinerea*, such as pectinases, cutinase and also glucanase and chitinase, through the action of protease secreted on plant surfaces; iii. competition for nutrients needed by the germinating conidia of *B. cinerea*; iv. changes in the *B. cinerea* hyphal surface chemistry that is responsible for attachment to the host surface, as detected by specific antibodies (Meyer et al., 2001); v. suppression of the oxidative burst that is caused by the attacking *B. cinerea* (Lapsker & Elad, 2001). Induced resistance was also described as a mode of action of T39 was proven in several plant – pathogen systems (De Meyer et al., 1998).

The interaction of this BCA with the phyllosphere population was only slightly investigated. It is crucial to study these components of biocontrol in order to further elucidate important features of the system and facilitate improved biocontrol with *T. harzianum* and probably with other BCAs. The objective of the research was to study the interaction of BCA (T39) with plants and with their phylloplane microflora. For this purpose we studied the effect of the treatments on the indigenous population that is culturable and we also examined changes in microbial communities of which genomic DNA was extracted and amplified so both cultivable and non cultivable microorganisms could be analysed.
Materials and methods

Conidia suspension of the fungus *T. harzianum* T39 (10^6/ml) and its powder formulation were applied to the same leaf as the pathogen or to the roots. *B. cinerea* infection was carried out with conidial suspensions (10^5/ml). Bean leaves were infected by 10-20 µl suspension drops. The plants were incubated under conditions of high humidity, 20-22ºC and 12 hours illumination. Disease severity was evaluated on a scale of 0-100 that was already developed for this purpose. Leaf samples were taken before and during incubation with the fungi in 1-2 days intervals.

Culturable microflora on leaf surfaces and inside the leaf tissue were studied following either surface washing in water containing 0.01% Tween 80 or surface leaf disinfection followed by maceration. Each of the microbial fractions were subjected to serial dilutions and conventional plating on PDA and nutrient growth media. Bacterial and yeast colonies were counted. Bacterial 16S ribosomal DNA (rDNA) extraction from bean leaves that were subjected to the various treatments was PCR amplified with the primers 530R and GC-338F. Amplicons were separated by size and base composition by denaturant gradient gel electrophoresis (DGGE) in order to fingerprint shifts in the structure of the natural plant-associated microbial communities (culturable and non-culturable) that may result from biocontrol and pathogen activity.

Discussion

Density Gradient Gel Electrophoresis (DGGE) is an acrylamide-based separation method in which mixed populations of nucleic acids can be separated not only on the basis of size, but also on their DNA composition. If PCR primers are chosen so that one end of the double stranded product contains a high number of guanine and cytosine (a GC clamp) and a linear gradient of urea is incorporated into an acrylamide gel, then a mix of PCR products applied to this gel will move through the matrix at a speed dependent on local melting of the DNA duplex due to the urea denaturant. Effectively this means that cDNA from 16S or 18S genes of genera, species and subspecies can be separated on a gel as discreet reproducible bands. These bands can then be cut out of the gel and sequenced. Comparison to the large rRNA databases can then provide a classification for all the species in a particular niche. Since RT-PCR is a semi-quantitative process, a time course experiment can reveal changes in the types and abundance of microbial populations such as leaf surfaces. Observation of the microbial dynamics associated with the application of a BCA using DGGE revealed a pronounced affect of the BCA on the population as a whole.

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Investigation of *Trichoderma* strains isolated from winter wheat rhizosphere

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**Abstract:** Species in the filamentous fungal genus *Trichoderma* are of great economic importance as sources of enzymes and antibiotics, plant growth promoters, degraders of xenobiotics, and most importantly, as commercial biofungicides. One hundred and forty *Trichoderma* strains were isolated from roots of winter wheat grown in agricultural fields of southern Hungary, and the identity of species was examined based on both morphological and molecular characters. The morphological data were collected by measuring structure and shape of conidiophores, phialides and conidia. For the investigation of molecular diversity, cellulose-acetate electrophoresis mediated isoenzyme analysis was applied. After initial testing of 12 enzymes for activity and resolution of bands, SIX of them (glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, 6-phosphogluconate dehydrogenase, peptidase A, peptidase B and phosphoglucomutase) proved to be appropriate for the analysis of the full sample set. Comparing the different electrophoretic types of the defined isolates, four of these enzymes could be used as molecular markers for the identification of FOUR *Trichoderma* spp.

**Key words:** taxonomy, isoenzyme analysis, cellulose-acetate electrophoresis, biocontrol, *Trichoderma*

**Introduction**

There is a worldwide need to adopt the practice of sustainable agriculture, using strategies that are environment-friendly, less dependent on agricultural chemicals and less damaging to soil and water resources. One of the key elements of such sustainable agriculture is the application of biocontrol agents for plant protection. *Trichoderma* species are filamentous fungi with teleomorphs belonging to the *Hypocreales* order of the *Ascomycota* division. Members of this genus are potent biocontrol agents, the proposed mechanisms resulting in biocontrol are including the stimulation of the defensive mechanisms of the plants (Benítez et al., 1998), competition for the substrate (Naár & Kecskés, 1998) as well as antibiosis by the production of antifungal metabolites and mycoparasitism by the action of cell-wall degrading enzymes (Benítez et al., 1998; Manczinger et al., 2000).

Identification of *Trichoderma* species based on morphological and cultural characteristics is sometimes difficult, because their traits exhibit variations on a continuous scale that may overlap between the species. The use of molecular markers for species-specific detection assays has become widespread. One of the methods, the cellulose-acetate electrophoresis mediated isoenzyme analysis has already been reported as useful and rapid method for the identification of *Ganoderma* (Smith & Sivasithamparam, 2000) and *Phytophtora* species (Oudemans & Coffey, 1991).
The aims of the present study were the isolation of *Trichoderma* strains from Hungarian soil samples, their morphological characterisation, and the detection of isoenzymes, which could be used routinely for their identification at the species level.

**Materials and methods**

**Isolation of Trichoderma strains from Hungarian soil samples**

The chopped roots of winter wheat were placed to Petri dishes containing selective medium (5 g/l peptone, 1 g/l KH₂PO₄, 10 g/l glucose, 0.5 g/l MgSO₄·7H₂O, 0.5 ml/l 0.2% dichloran-ethanol solution, 0.25 ml/l 5% Rose Bengal, 20 g/l agar). Growing *Trichoderma* strains were transferred from the plates onto yeast extract medium (2 g l⁻¹ yeast extract, 5 g l⁻¹ KH₂PO₄, 20 g l⁻¹ agar). All isolates were grown from single conidia for identification. The taxonomic identity was examined according to an interactive key available for the genus *Trichoderma* at the web site of the United States Department of Agriculture, Agricultural Research Service (http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm).

**Cellulose acetate electrophoresis**

Inoculation of 250 ml liquid yeast extract broth media was performed with conidial suspensions to a final concentration of 10⁶ conidia m/l. Cultures were incubated for four days on an orbital shaker at 200 rpm and 25 °C. Mycelia were filtered through filter paper, washed three times with distilled water and lyophilized. Dried mycelia were grinded to dust with a pestle in a mortar. Protein extraction was performed according to the method described by Láday & Szécsi (2001). Cellulose acetate electrophoresis (CAE) was conducted as described by Hebert & Beaton (1993), the CAE system was purchased from Helena Laboratories (Beaumont, TX). Titan III cellulose acetate gels were soaked for at least 30 min in electrophoresis buffer (0.25 mmol Tris-glycine, pH 8.5) in a bufferizer and blotted dry between sheets of filter paper. The protein extracts were applied from the sample plate to the gel with a Super Z-12 Applicator. Electrophoresis was carried out at 180 V for 20 min. Gels were stained for 12 enzyme systems (Table 1), enzyme activities were detected using agar overlays (Hebert & Beaton, 1993). The relative mobility (R_f value) of each isoenzyme band was calculated using the anodally moving band of *T. harzianum* T66, which was arbitrarily chosen as the standard. For each enzyme assay, bands were designated by the abbreviations of enzymes and the percentage mobility of the band relative to the standard band.

From the isoenzyme data, matrices were created based on the presence or absence of a given activity band. Simple matching coefficients were calculated with the PHYLTOOLS software package (version 1.32). Phylogenetic trees were prepared by the neighbor-joining method (Saitou & Nei, 1987) using the program NEIGHBOR of the PHYLIP software package, version 3.57c, (Felsenstein, 1995). Data were analyzed using the outgroup rooting method with a *Neosartorya glabra* strain designated as the outgroup taxon.

**Results and discussion**

One hundred and forty *Trichoderma* strains were isolated on selective media from roots of winter wheat from thirty-one test holes of six agricultural fields in southern Hungary.

From the isolates, 79 were identified based on morphological characters of conidiophores, phialides and conidia, and colony radius on both potato dextrose agar and corn meal agar. These strains were included in species *T. viride* (34 strains), *T. virens* (32 strains), *T. longibrachiatum* (5 strains) and *T. atroviride* (8 strains). *T. virens* was assigned to section *Pachybasium* (Sacc.), *T. viride* to section *Trichoderma*, while *T. longibrachiatum* and *T.
atroviride to section Longibrachiatum. The undefined isolates are also belonging to the Trichoderma genus, but their exact morphological analysis is still in progress.

Table 1. Enzyme systems tested and their Enzyme Commission (E.C.) numbers

<table>
<thead>
<tr>
<th>Enzyme System</th>
<th>Abbreviation</th>
<th>E.C. no.</th>
<th>Activity</th>
<th>No. of bands with different Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>6PGDH</td>
<td>1.1.1.44</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>Aconitase</td>
<td>ACN</td>
<td>4.2.1.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>G6PDH</td>
<td>1.1.1.49</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>Glucose-6-phosphate isomerase</td>
<td>GPI</td>
<td>5.3.1.9</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>Glycerol-3-phosphate dehydrogenase</td>
<td>GDH</td>
<td>1.1.1.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>MDH</td>
<td>1.1.1.37</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>PRX</td>
<td>1.11.1.7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peptidase A (Gly-Leu)</td>
<td>PEP A</td>
<td>3.4.11/13</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>Peptidase B (Leu-Gly-Gly)</td>
<td>PEP B</td>
<td>3.4.11/13</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>PGM</td>
<td>5.4.2.2</td>
<td>+</td>
<td>11</td>
</tr>
<tr>
<td>Shikimate dehydrogenase</td>
<td>SKDH</td>
<td>1.1.1.25</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>SUD</td>
<td>1.3.99.1</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Twelve enzyme systems were tested initially for activity, resolution, and consistent appearance of the bands. Six enzymes which showed clear, reproducible banding patterns were selected for the full sample set, from which 42 distinct bands were scored and used in the analysis (Table 1). A single band common to all isolates was detected for GPI, PEP A, PEP B and PGM enzyme systems. Electromorphs of G6PDH were double and triple banded in the isolates observed.

All six enzymes were polymorphic for the full sample set, PGM, G6PDH, PEP B and 6PGDH showed interspecific, while GPI and PEP A showed intraspecific polymorphisms. The patterns of the enzymes were used to group the defined strains into different electrophoretic types (ETs). With this grouping we obtained 25 different ETs. In the case of the 79 defined isolates the patterns of enzymes fell into seven common ETs: ET I, ET II and ET III for T. viride, ET IV and ET V for T. virens, ET VI for T. atroviride and ET VII for T. longibrachiatum. In the cases of T. virens and T. viride, GPI and PEP A patterns split the species to the mentioned ETs, while PGM, G6PDH, PEP B and 6PGDH patterns proved to be appropriate to identify the four species.

When a dendrogram was created from the diagnostic isoenzyme data, the whole sample set clustered in eleven major groups and the morphologically characterised strains clustered in four distinct groups according to the corresponding species (Figure 1). The phylogenetic distance between species is in correlation with taxonomic categories, however, the final phylogenetic analysis requires more isoenzyme data.

There is a great number of different enzyme systems potentially available for use in isoenzyme analysis, therefore it is highly probable that an excellent molecular marker can be found for the differentiation and practical diagnostics of different Trichoderma species. Based on the results of the present study, cellulose acetate electrophoresis mediated isoenzyme analysis seems to be promising method for the rapid and accurate identification and classification of Trichoderma isolates.
Figure 1. Neighbour joining dendrogram resulting from the analysis of the diagnostic isoenzymes of the defined isolates with the different electrophoretic types

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References


Survey of antagonistic yeasts occurring in apple trees managed with different production systems

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Apples are grown in Brazil using conventional, integrated and organic production systems. Since different management practices could affect the beneficial microflora growing as epiphytes on the crop, this research aimed to compare the frequency of yeasts which are antagonistic to the two main apple pathogens: Colletotrichum gloeosporioides and Penicillium expansum. Yeasts with antagonistic properties against the first pathogen were surveyed in the three production systems and those antagonistic to the second one, were obtained from apple trees growing in the conventional and in the integrated system. The isolates were selected from fruit washings and morphologically characterized. Selection of antagonists was done by spraying unwounded apples with a suspension containing both the pathogen and each yeast isolate when C. gloeosporioides was used or on wounded apples when P. expansum was studied. Results showed that in both the integrated and ecological systems, the frequency of yeasts antagonistic to C. gloeosporioides was similar and higher than in the conventional system. Yeasts antagonistic to P. expansum were also more frequent in integrated than in conventional system. Many antagonistic organisms were selected with potential to control both pathogens suggesting that yeasts must be protected on apple orchards.
Strategy to control *Verticillium dahliae* in oilseed rape using *Serratia plymuthica* HRO-C48

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**Abstract:** *Verticillium dahliae* Kleb. is a soil borne fungus causing vascular disease that results in severe yield and quality losses in many crops. In the last years yield losses in oilseed rape production caused by *V. dahliae* became of alarming relevance worldwide. Till now, there exists no possibility to control the pathogen due to the lack of potent fungicides, and the ecological behaviour of the pathogen. In previous studies, the effect of *Serratia plymuthica* strain HRO-C48 to control *V. dahliae* and to promote plant growth of strawberry was shown. The objective of this study is to develop a strategy to use the biocontrol capacity of *S. plymuthica* for protecting oilseed rape against Verticillium wilt. Practically, pelleting, film coating and seed priming are possibilities to apply *S. plymuthica* cells on oilseed rape seeds. Using these treatments, cell counts of log$_{10}$ 6 to 7/seed were achieved. *Serratia* treated seeds were used to evaluate rhizosphere competence of *S. plymuthica* in greenhouse trials. Rifampicin resistant cells of *S. plymuthica* were re-isolated from the rhizosphere in abundances of about 5 x 10$^4$ cfu/g root. The form of neither treatment nor the initial cell density had an impact on the establishment in the rhizosphere.

**Key words:** *Verticillium dahliae*, *Serratia plymuthica*, oilseed rape, seed inoculation, rhizosphere competence

**Introduction**

Verticillium wilt caused by the soil borne fungus *Verticillium dahliae* Kleb. is of increasing significance world-wide, especially in crops and in tree hosts such as olives (Tjamos et al., 2000). Formerly, methylbromide was used to desinfect *Verticillium* microsclerotia containing soil. These and other related substances show highly toxic effects to many non-target organisms, and an additional negative influence on the world climate. Therefore, in many countries it is still forbidden and will be phased out till 2005 worldwide. The beneficial rhizosphere microbiota can be managed for increased plant health by the introduction of beneficial microorganisms, and therefore offers an environmentally friendly alternative to control Verticillium wilt. *S. plymuthica* strain HRO-C48 (German Collection of Microorganisms and Cell Cultures: DSMZ 12502) 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 the beneficial Gram-negative bacterium *Serratia 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* and to enhance fruit yield. A product called RhizoStar® for commercial strawberry production was developed (Berg et al., 1999). The experience will be used to develop a successful model for oilseed rape. Therefore, optimal formulation and application methods will be developed.
Material and methods

Seed inoculation
Rifampicin resistant mutant of *Serratia plymuthica* strain HRO-C48 was cultured in 500 ml Erlenmeyer flask containing 200 ml nutrient broth. Inoculated medium was incubated for 24 h at 30°C and 150 rpm. Cells were separated by centrifugation at 7500 g and resuspended in a medium according to the application methods. All suspensions were adjusted to log10 10 cfu/ml Using pelleting 500 µl of cells suspended in 1.5% methyl cellulose solution added to 2.0 g of seeds (cv. Ural, NPZ, Hohenlieth, Germany), which were rotating in a self-made pelleting machine. To bind remaining liquid, 2.0 g talcum was added. Seeds stayed in the machine until the coating was uniform. Using film coating 0.4 g of seeds was wetted in 1.0 ml of cell suspension containing 1.0% sucrose. Afterwards inoculated seeds were lyophilisated for 5 h. For seed priming 0.4 g of seeds were incubated for 18 h at 20°C in 2.0 ml of cells suspension containing 0.9% sodium chlorine. During incubation seeds were agitated. Infiltrated seed were dried for 24 h at 20°C to the desired moisture content of about 5%.

Greenhouse experiments
Treated seeds were sown in pots with a volume of 250 ml. Soil mixed with 20% vermiculite was used. Plants grew for 30 days at a temperature range from 20°C to 30°C. They were watered every second day. Roots with adhering soil from three plants were sampled into sterile bags. To extract the rhizosphere microorganisms from the roots 50 ml of de-mineralized water were added, and samples were homogenized in a Stomacher laboratory blender for 180 s (BagMixer, Interscience, St. Nom, France). Samples were serially diluted with sterile 0.85% NaCl and and plated onto NA medium (Sifin, Berlin, Germany) containing 100 ppm Rifampicin (Fluka, Buchs, Switzerland). Plates were incubated for 24 h at 30°C and colony forming units (cfu) were counted to calculate the means of colonies (log10 cfu) based on fresh weight.

Results and discussion
Two trials were carried out to evaluate the ability of *S. plymuthica* HRO-C48 to colonize the rhizosphere of oilseed rape, and to suppress the pathogen under greenhouse conditions. In greenhouse trial I, different application procedures were evaluated (Figure 1). Three different types of treatments were selected. Although all original suspensions used for treatments were adjusted to log10 10 cfu/ml the treatment resulted in different cell counts per seed. Using pelleting and film coating about log10 7 cfu/seed could be re-isolated from the seed surface. Using seed priming, *Serratia* cells were infiltrated, and colonized the endosphere of seeds. Cells of *S. plymuthica* were found inside the seeds about log10 6 cfu/seed. All treated seeds were used to evaluate rhizosphere competence of *S. plymuthica* in greenhouse. After 30 days, rifampicin resistant cells of *S. plymuthica* were re-isolated from the rhizosphere in abundances of log10 4.69 ± 0.081 cfu/g root.

In greenhouse trial II, different concentrations of *Serratia* cells were evaluated (Figure 2). Plants were treated with five different concentrations log10 3 - 7 cfu/seed using film coating. Surprisingly, after 30 days all treatments resulted in the same concentration of *Serratia* cells on roots of log10 4.66 ± 0.045 cfu/g root. In former studies, a dose-dependent effect on *Serratia* treated strawberry seedlings was found (Kurze et al., 2001). Further inoculation studies are necessary to manage *Serratia* in the rhizosphere of oilseed rape.
Figure 1. Colonisation of the rhizosphere of oilseed rape by *Serratia plymuthica* HRO-C48 using different application procedures, and with suspension at a concentration of $\log_{10} 10$ CFU ml$^{-1}$. Cells were re-isolated on NA after 30 days.

Figure 2. Colonisation of the rhizosphere of oilseed rape by *Serratia plymuthica* HRO-C48 using different cell concentrations per seed. *S. plymuthica* was applied by film coating. Cells were reisolated on NA after 30 days.

Concluding, the form of neither treatment nor the initial cell density had an impact on the establishment of *Serratia plymuthica* HRO-C48 in the rhizosphere of oilseed rape.

**Acknowledgement**

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Molecular characterization of biocontrol agents

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Abstract: Genetic variability within 69 biocontrol isolates of Trichoderma, collected from different geographical locations and culture collections and selected as biocontrol agents, was studied. Sequence data obtained from the internal transcribed spacer 1 (ITS1) region of ribosomal DNA and a fragment of the translation elongation factor 1 (tef1) gene were used to undertake a phylogenetic analysis. More than 50% of the potential biocontrol strains were grouped within Trichoderma section Pachybasium. Of these, 81% were grouped within the cluster that included the ex-type strains of T. harzianum and T. inhamatum and 16% were grouped with T. virens. Within T. sect. Trichoderma, which included 36% of the 69 strains, 56% were grouped with T. asperellum and 24% with T. viride, T. atroviride or T. koningii.

Key words: biological control, Trichoderma, internal transcribed spacer (ITS), translation elongation factor (tef)

Introduction

Utilizing microorganisms to combat plant disease is the basis of biocontrol. Strains of the genus Trichoderma have been shown to efficiently control several phytopathogenic fungi (Monte, 2001). The mechanisms of control previously suggested have included antibiosis, mycoparasitism, and competition for nutrients. The capacity to promote growth and induce resistance in plants are activities which have also been described for members of this genus.

Initially, nine species aggregates based on morphological features were distinguished by Rifai (1969). However, this classification has been considered preliminary since some aggregates comprised morphologically indistinguishable species and formed complexes. Molecular data derived from enzymes and DNA have been useful to characterize Trichoderma more objectively than traditional methods (Lieckfeldt et al., 1998). The most important biocontrol species are T. harzianum, T. virens (formerly Gliocladium virens) and T. viride. Molecular data have shown a high level of heterogeneity in these and other species within the genus. The heterogeneity of T. harzianum has been described (Gams & Meyer, 1998; Hermosa et al., 2004; Samuels et al., 2002). The present study was undertaken to determine the genetic diversity existing in a collection of 69 strains selected as biocontrol agents against phytopathogenic fungi.

Materials and methods

Isolates
A total of 69 isolates of Trichoderma spp., associated with biological control, were analyzed. The geographical origin of these isolates included: 22 from Spain, 11 from Brazil, 8 from the
United Kingdom, 7 from India, 6 from Colombia, 4 from France, 2 from Argentina and 1 each from Antarctica, Australia, the Czech Republic, Philippines, Slovakia, Sri Lanka, U.S.A. and Zimbabwe. Of these, 38 were isolated by our group, 16 had been included in previous studies (Grondona et al., 1997; Hermosa et al., 2000), 14 were obtained from the several culture collections, and one isolate was from an unknown location. Thirty-four of the 38 *Trichoderma* strains isolated by our group were obtained from soil using a soil dilution plate method.

**DNA extraction, amplification and sequencing**

Total DNA was extracted using the method described by Raeder & Broda (1985). The ITS1 region of the nuclear rDNA gene cluster was amplified using primers ITS1 and ITS2 (White et al., 1990) for 53 isolates. A 0.2 kb fragment of *tef1* gene was amplified, using the primers tef1fw (5'-GTGAGCGTGTTTATCACCA-3') (O'Donnell et al., 1998) and tef1rev (5'-GCCATCCCTTGGAGACCAGC-3') (Kullnig-Gradinger et al., 2002) for 25 isolates. PCR were performed in a total volume of 50 µL containing 25 ng of genomic DNA and 0.20 µM concentrations of each primer described above. The amplification program included an initial denaturalization cycle of 5 min at 94ºC, followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC (for ITS1 region) or 59ºC (for *tef1* fragment), 1 min at 72ºC, and a final extension step of 7 min at 72ºC in a Perkin-Elmer Cetus 9600 thermal cycler. Following amplification, the PCR products were electrophoresed on 1% agarose gels buffered with 1X TAE (Sambrook et al., 1989) and stained with ethidium bromide. All PCR products were purified and sequenced in both directions using the amplification primers.

**Molecular analyses**

Sequence alignments were generated using the CLUSTALX 1.81 programs (Thompson et al., 1997). Published ITS1 and *tef1* sequences for strains included within sections *Trichoderma*, *Pachybasium* and *Longibrachiatum* were retrieved from GenBank and included in the alignments. Alignments were manually edited and optimized using GeneDoc. Phylogenetic analysis was carried out with the program PAUP (Phylogenetic Analysis Using Parsimony, Version 4.0, Sinauer Associates, Sunderland, Mass.). Neighbor-Joining (NJ) trees were constructed for each data set (ITS1 or *tef1*) using the Kimura-2-parameter distance measure (Kimura, 1980). *Hypocrea aureoviridis* CBS 245.63 was used as outgroup. The robustness of the internal branches was assessed with 1000 bootstrap replications.

**Results and discussion**

The ITS1 sequence of 53 *Trichoderma* isolates ranging from 180 and 220 bp was obtained and analysed. An initial alignment of the ITS1 sequences from 69 biocontrol isolates identified 21 distinct sequence types. Alignment and comparison of the ITS1 region of these 21 sequences with 26 known species or genotypes deposited in GenBank and representing the four sections of *Trichoderma*, identified 33 distinct sequence types. The NJ tree obtained from analysis of these 33 distinct sequences types was similar to the tree generated using an alignment containing the ITS1 sequences from all isolates. The biocontrol isolates were distributed within three of the four sections of the genus *Trichoderma*. Of the 69 biocontrol strains, 53.6% (37 isolates) were located in *T. sect. Pachybasium*, 36.2% (25 isolates) in *T. sect. Trichoderma* and 10.14% (7 isolates) in *T. sect. Longibrachiatum*. The separation of these sections was supported by bootstrap values of 65, 100 and 100%, respectively.

The alignment, comparison and analysis of a 150 bp sequence of the intron between exon 5 and 6 of the *tef1* gene from 25 strains, representing 17 of the distinct groups. Analysis of the *tef1* sequence distributed the 25 biocontrol strains as following: 8 strains in *T. sect. Pachybasium*, 14 strains in *T. sect. Trichoderma* and 3 strains in *T. sect. Longibrachiatum*. 
In a broad sense, the topologies of the trees generated using ITS1 and tef1 data were similar but not identical. Some biocontrol strains had different grouping in ITS1 or tef1 trees. Another study also noted that phylogenies based solely on ITS data do not necessarily have the same topology as that made from multiple gene (Kullnig-Gradinger et al., 2000). Based on these results, the high degree of variability within the tef1 marker also allowed the separation of species within T. sect. Trichoderma, since T. atroviride, T. viride and T. koningii were clearly differentiated. Lieckfeldt et al., (1999), using ITS sequence data and biochemical features, and Hermosa et al., (2000), using ITS1 sequences, also observed that T. atroviride was more closely related to T. koningii than with T. viride. Within T. sect. Trichoderma, two strains formed an independent clade from T. viride and H. cf. rufa strains, and could be members of an undescribed species. Similarly, another isolate, which was neither T. hamatum nor T. pubescens, was separated from these species using the tef1 marker. Two groups were observed within the T. asperellum clade using tef1 data. In general, this grouping was similar to that observed in the ITS1 NJ tree, and was in agreement with other studies (Hermosa et al., 2001; Lieckfeldt et al., 1999). The distribution of species within T. sect. Longibrachiatum was coincident in both ITS1 and tef1 trees.

Based on the topologies observed within the two NJ trees it was confirmed that the ITS1 marker had less capacity than tef1 to resolve taxa. Since the ITS1/tef1 phylogram (data not shown) was more similar to the tef1 phylogram, despite only representing a portion of the 69 isolates, this confirmed the taxonomic value of the tef1 gene described by other authors (Chaverri et al., 2003; Kullnig-Gradinger et al., 2002; Samuels et al., 2002).

One aim when isolating Trichoderma is to identify strains that can be used in new agricultural applications, and at present several are either being or have been registered as fungicides in various countries. Among the many requirements for registration, it is necessary to identify, characterize and assign names to the strains.

References


Studies of soil and rhizosphere bacteria to improve biocontrol of avocado white root rot caused by *Rosellinia necatrix*

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**Abstract:** In this work we present the results from isolations of soil bacteria and rhizosphere bacteria from avocado orchards located in Andalucía (southern Spain) and the studies of their antagonistic activity. Biocontrol of white root rot caused by *Rosellinia necatrix* was tested as well. Different strains have been selected with antagonistic activity against this pathogen. Antagonists were also active against the world-wide pathogen *Phytophthora cinnamomi* and also against both pathogens. Bioassays with avocado plants have been initiated in order to select bacteria with biocontrol capacity.

**Key words:** *Persea americana*, *Phytophthora cinnamomi*, biological control, soil borne fungi

**Introduction**

White root rot that is caused by *Rosellinia necatrix* Prill. (asexual phase *Dematophora necatrix* Hartig) is a very destructive disease in many different crops (Khan, 1959). In Spain, the fungus was described during the 1980ies, and nowadays the avocado white root rot is one of the two most important diseases in avocado orchards in the Andalusian area. The other main disease is root rot that is caused by *Phytophthora cinnamomi* Rand (López Herrera, 1989).

*R. necatrix* is an aggressive fungus which attacks secondary roots. It develops under the cortex by mycelial aggregations which invade the root system. On avocados, aerial symptoms of infection are visible only when the whole rot system is colonized by the fungus; at this time, the tree suddenly collapses and dies. Disease control includes different methods; irrigation control, isolation of affected trees and the use of pathogen-free material. Soil solarization is effective in its control and some results by soil applications of different fungicides under greenhouse conditions have been reported. Actually, biological control of this disease is under development through evaluations of different avocado germplasm against the fungus in order to obtain tolerant rootstockst (Pérez Jiménez et al., 2003). At the same time, a selection of biological control agents with different biocontrol mechanisms including; micoparasitism, antibiotic production or competence, are under development (Cazorla et al., 2001; Ruano et al., 2003). The objective of this study is to improve the biological control of avocado white root rot by isolating and analysing soil and rhizobacterial strains as potential biocontrol agents.
Material and methods

Bacterial isolations
Bacteria were isolated from soil and root samples collected from different asymptomatic avocado trees, infected or not with R. necatrix, located in Andalusia (Table 1). Samples were manipulated in different ways in order to isolate soil or rhizosphere bacteria. Root samples were washed under water and macerated in phosphate buffer 0.1 M for 5 min and soil samples were suspended in phosphate buffer 0.1 M, pH 7 and shaken for 2 h. The subsequent suspensions were serially diluted onto King’s B agar plates. After 48 h at 24ºC the colonies were counted and simultaneously, different colonies based on different morphological characteristic, were transferred to new KB-agar plates to obtain pure cultures. Purified isolates were routinely stored at -80ºC on LB medium supplemented with 25% glycerol.

Antagonism test
Bacterial isolates were screened for in vitro antagonism toward R. necatrix (isolate CH253) or P. cinnamomi (isolate CH14). These pathogenic fungal isolates had been previously characterized as high virulent isolates. Bacterial isolates were tested on KB-agar plates against R. necatrix and on PDA plates against P. cinnamomi. For each pathogen, a PDA disc (0.5 cm diameter) was taken from the margins of 7 days growing fungal colony and placed in the centre of a petri dish. Furthermore, four bacterial isolates were placed around the disc at a distance of 2 cm. After 7 days at 25ºC, the inhibition zones of fungal mycelia around bacterial colony were discerned. Bacterial isolates with antifungal activity were tested again alone in the plate assay.

Biocontrol test
Preliminary biocontrol test were preformed using avocado plants cvs Topa Topa obtained by in vitro germinated embryos (Pliego-Alfaro et al., 1988). Roots from two month-old plants were disinfected in 0.05% sodium hypochlorite for 10 min and later dipped in a suspension of each bacterial isolate (10^9 cfu.ml^-1) for 15 min. The bacterized plants were planted into 250 ml pots with a substrate composed of peat, coir fibre and perlite (v:v:v 10:2:1). Pots were inoculated with wheat seeds (4 g/l substrate) infected with R. necatrix (Sztejnberg et al., 1987).

Two antagonistic rhizobacterial isolates to R. necatrix and P. cinnamomi were assayed (O4.39 and O5.58). Additionally we test two rhizosphere-competent strains (GBF.1.5 and GBF.1.11) using a different methodology (Pliego et al., 2004) and the rizhobacterium PCL1606, previously selected for its biocontrol of R. necatrix by Cazorla et al. (2001). Plants infected with only the pathogen and not infected were used as controls. For each experiment ten replications were used. The plants were grown at average temperatures of 25ºC, artificial light (4000 lux) and 80% relative humidity. A disease severity rate with four different levels was established considering aerial symptoms: healthy, wilting, advanced wilting and death. The standardized areas under the disease progress curve for each isolate were calculated and compared by ANOVA (Campbell & Madden, 1990).

Results and discussion

Bacterial isolates and antagonistic test
Two hundred and twenty four bacterial isolates were selected from root and soil samples coming from 11 avocado trees located in six different orchards, bacteria populations were slightly higher in soil (10^6-10^7) than in rhizosphere (10^5-10^6). From these isolates, 63.4% were gram negative small rod shaped bacteria and 36.6% were gram positive rod shaped (Table 1).
Simultaneously, we selected more antagonistic bacteria against *R. necatrix* (13.4%) than against *P. cinnamomi* (6.7%), and 5.4% of the analyzed isolates were antagonistic to both pathogens. From the antagonistic isolates to *R. necatrix*, 56.6% were gram negative and 41% of these were fluorescent, which indicates that could be included into the *Pseudomonas* genera (Table 1).

**Table 1. Details of sampled avocado orchards and trees in the Andalusian area and antagonistic ability shown by selected bacteria isolated from rhizosphere or soil**

<table>
<thead>
<tr>
<th>Orchard</th>
<th>Tree</th>
<th>Pathogen</th>
<th>Selected isolates</th>
<th>Antagonistic isolates to Rn (^1)</th>
<th>Antagonistic isolates to Pc (^2)</th>
<th>Antagonistic isolates to Pc and Rn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>N°</td>
<td>Gram - F (^3)</td>
<td>N°</td>
<td>Gram - F</td>
</tr>
<tr>
<td>O1</td>
<td>O1.1</td>
<td>Rn</td>
<td>38</td>
<td>28</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>O1.2</td>
<td>N(^3)</td>
<td>20</td>
<td>14</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O1.3</td>
<td>N</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>O2</td>
<td>O2.1</td>
<td>Rn</td>
<td>12</td>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O2.2</td>
<td>N</td>
<td>14</td>
<td>8</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O2.3</td>
<td>N</td>
<td>13</td>
<td>8</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>O3</td>
<td>O3.1</td>
<td>Rn</td>
<td>21</td>
<td>12</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>O4</td>
<td>O4.1 Rn/Pc</td>
<td>39</td>
<td>25</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>O4.2</td>
<td>N</td>
<td>15</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>O5</td>
<td>O5.1</td>
<td>N</td>
<td>22</td>
<td>13</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>O6</td>
<td>O6.1 N</td>
<td>10</td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td>224</td>
<td>142</td>
<td>44</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^1\) Rn: *R. necatrix* isolated from sampled tree. \(^2\) Pc: *P. cinnamomi* isolated from sampled tree. \(^3\) N: no pathogen detected on sampled tree. \(^4\) F: fluorescent bacteria.

**Biocontrol test**

Between tested isolates, isolate O5.58 was statistically different with the smallest standardized area under the disease progress curve (Figure 1).

**Figure 1.** Analize ANOVA for standarized areas under the disease progress curve for each treatment (values with different letter indications denote a statistically significant difference).
Under our experimental conditions, in this complex system, one isolate (Figure 1) showed significant reduction of the disease symptoms. The rest of the strains analyzed did not show significant differences. These results indicate that the spectrum of environmental conditions for the development of biocontrol bacteria must be analyzed in order to establish the optimum conditions in which this bacteria exhibit their biocontrol capacity. Experiments have to be repeated and new evaluations of the rest of our bacterial collection with this avocado test system has to be done.

Acknowledgements

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References

Occurrence of breakdown in the biocontrol of crown gall disease by the *Agrobacterium radiobacter* strain K84 in Italy

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Abstract: Biological control of crown gall by using the *Agrobacterium radiobacter* strain K84 represents the most successful application of biological methods for preventing plant diseases worldwide. However, the efficacy of this biocontrol method may be reduced by the selection of pathogenic recombinants that are insensitive to the antagonist. Dangerous recombinants take origin through plasmid exchange that may occur between the antagonist and tumorigenic agrobacteria. In 2001 a breakdown in the biocontrol of crown gall disease was reported in an Italian nursery of peach plants where K84 was used to protect the rootstocks. Agrobacteria strains were isolated from the tumors and from the soil around the tumors (tumorosphere) of the galled plants and identified. Molecular studies were performed to determine the plasmid content and the chromosomal characteristics of the isolates in order to identify the type of plasmid exchange and the bacterial genotypes involved in the failure of the biocontrol agent. Transconjugant strains were isolated both from gall tissues and from tumorosphere and all were originated by the transfer of pAgK84 from K84 strain to virulent and avirulent autochthonous agrobacteria. The analysis of pathogenetic characteristics and root colonization ability of recombinants showed that these strains are very competitive in soil and may represent a real threat to the application of K84.

Key words: biocontrol, K84, transconjugants, crown gall

Introduction

Crown gall is a dangerous disease caused by the soil-borne bacterium *Agrobacterium tumefaciens*. Disease induction is due to the transfer of a DNA fragment (T-DNA) containing the oncogenic genes, from the tumor inducing plasmid (pTi) of tumorigenic agrobacteria to the plants where it is integrated and expressed (Chilton et al., 1977). Crown gall is effectively prevented by treating the plant before transplanting with a suspension containing the antagonist *Agrobacterium radiobacter* strain K84 (Kerr and Htay, 1974). Biocontrol by strain K84 is a complex phenomenon involving different mechanisms but is mainly performed through the production of a bacteriocin, the agrocin 84, that is coded by the plasmid pAgK84. This plasmid also carries the genes codifying for pAgK84 conjugal transfer and agrocin 84 immunity (Farrand et al., 1985).

The repeated use of K84 can lead to the appearance of dangerous Agrobacterium cells harbouring both pAgK84 and pTi that are originated by plasmid exchanges between K84 and tumourigenic strains. These transconjugants may be responsible for the breakdown of biocontrol of crown gall (Panagopoulos et al., 1979).

A three year study was performed to determine the efficacy and the stability of K84 in nine nurseries located in Southern Italy that were using the antagonist to protect peach rootstocks. In one nursery a breakdown in the biocontrol of crown gall occurred.
**Materials and methods**

**Agrobacteria isolation and identification**

Agrobacteria were isolated from tumors developed on peach rootstocks that were treated in preplanting with a suspension of K84 strain and from the soil around tumors (tumorosphere). Isolation and identification of agrobacteria were performed following the procedure of Moore et al., (1988). All agrobacteria were tested for pathogenicity on tomato (*Lycopersicon esculentum*) and datura (*Datura stramonium*), for opine catabolism and for *in vitro* sensitivity to agrocin 84.

**Characterization of transconjugants**

All strains identified as *A. tumefaciens* were tested by colony hybridization with *tmsI*-tmr (from the T-DNA region of pTi) and *agn* probes (from the region codifying for agrocin 84 synthesis of pAgK84). Strains that were considered putative transconjugants were further analyzed for plasmid profile and *in vitro* agrocin production. Molecular analysis of transconjugant strains by PCR-RFLP of the 16S and the Intergenic spacer between 16S and 23S was performed in order to identify the ribotypes involved in the plasmid exchanges, since the variability of this region allows the identification of different strains within the *Agrobacterium* species (Ponsonnet & Nesme, 1994). CfoI, HaeIII, NdeII and TaqI restriction enzymes were used to digest the 2700 bp amplification products obtained by using FGPS6 and FGPL132’ primers (Ponsonnet & Nesme, 1994). Representative transconjugant strains were also tested for their ability to colonize plant roots and to induce tumors on peach plants in order to determine the risks related to occurrence of these recombinants in the field.

**Results**

Six-hundred and seventy-eight isolates from tumors were identified as *Agrobacterium tumefaciens*, 382 of which were pathogenic on tomato and datura. Results of the tests performed to identify putative transconjugants compared with those obtained for strain K84 showed that 24 virulent and 18 avirulent strains hybridized with *agn* probe, produced agrocin 84 in vitro and were insensitive to it; 35 other by 2 avirulent strains, that resulted positive at the hybridization with *agn* probe, were considered isolates of K84 (Table 1).

<p>| Table 1. Characteristics of <em>Agrobacterium</em> strains isolated from peach tumors that hybridized with <em>agn</em> probe. *strains unable to catabolize mannopine, nopaline and octopine |</p>
<table>
<thead>
<tr>
<th>Strains</th>
<th>Pathogenicity</th>
<th>Biovar</th>
<th>Agrocin sensitivity</th>
<th>Opine catabolism</th>
<th><em>tmsI</em>-tmr</th>
<th><em>agn</em></th>
<th>Agrocin 84 production</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>+</td>
<td>1</td>
<td>–</td>
<td>nopaline</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>–*</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>2</td>
<td>–</td>
<td>–*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>2</td>
<td>–</td>
<td>nopaline</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>35</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>nopaline</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K84</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>nopaline</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Out of 900 agrobacteria isolated from tumorosphere, 15 tumorigenic by 2 strains were positive at the hybridization with *agn* probe, produced agrocin 84 *in vitro* and were insensitive to it. They were then considered putative transconjugants. Analysis of plasmid profile of putative transconjugants isolated from tumor and tumorosphere showed that all strains harboured a plasmid of the same size as pAgK84 (Figure 1).

![Figure 1. Plasmid profiles of transconjugant strains](image)

Southern blotting with the *agn* probe definitively detected the pAgK84 in the bacterial cells. Molecular analysis of transconjugant strains by PCR-RFLP of the 16S plus IGS showed that all transconjugant ribotypes were different from that of K84 and than that the pAgK84 was transferred from the antagonist to autochthonous virulent and avirulent strains.

Plant root colonization ability of K84, of a transconjugant and a tumorigenic non recombinant strains was determined *in vitro* on tomato plantlets grown in tubes containing suspensions of the different combinations of strains. Results showed that root colonization by the non recombinant strain was strongly reduced in presence of the antagonist K84 while the colonization ability of transconjugant strain was not affected by K84 (data not shown).

Field biocontrol trials performed by inoculating peach rootstock plants showed that disease incidence induced by transconjugant and a tumorigenic non recombinant strains were similar, and that disease induction by the transconjugants was not prevented by treating the plants with the biocontrol strain K84 (Table 2).

<table>
<thead>
<tr>
<th>Disease incidence (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Non transconjugant strain (NT)</td>
<td>90.3 a</td>
</tr>
<tr>
<td>Transconjugant (T)</td>
<td>94.4 a</td>
</tr>
<tr>
<td>NT + K84</td>
<td>3.6 b</td>
</tr>
<tr>
<td>T + K84</td>
<td>82 a</td>
</tr>
</tbody>
</table>

**Discussion**

Transconjugant *Agrobacterium* cells originated from the transfer of pAgK84 from the biocontrol strain K84 to tumorigenic and nontumorigenic agrobacteria were found in tumors and tumorosphere of galled peach plants grown in a nursery of Southern Italy. This represent
the first report of a breakdown in the biocontrol of crown gall by K84 in Europe in a commercial nursery. The occurrence of dangerous transconjugants following the use of K84 has been demonstrated experimentally in several instances (Panagopoulos et al., 1979; Stockwell et al., 1996; Vicedo et al., 1993). Results of this study show that the pAgK84 was transferred from the antagonist to tumorigenic and non-tumorigenic soil-borne agrobacteria. Appearance of dangerous transconjugants occurred only in one out of the nine nurseries considered in this study where high disease incidence was also observed (15%). The antagonist K84 resulted indeed highly effective in protecting each rootstocks in the other eight nurseries considered in this study, where only few plants resulted affected by crown gall (0.01% in the average). Tumorigenic transconjugants were highly competitive in the rhizosphere, were able to induce high disease incidence and were not controlled by K84 in the field. These data sharply show that they may represent a real threat to the use of K84.

To completely prevent the risk of transconjugants selection related to the use of the biological control method, a Tra- derivative of K84 unable to transfer the plasmid has been selected (Jones et al., 1988). This strain, called K1026, has been obtained by the deletion of the region codifying for pAgK84 transfer, and no foreign DNA has been inserted. However, its use is now allowed in Australia, New Zealand but not in Europe where the current legislation consider the K1026 strain a genetically modified microorganisms.

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Microorganisms associated with *Platanus acerifolia* W. growing in areas infected by *Ceratocystis fimbriata* f. sp. *platani*

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Abstract: *Platanus acerifolia*, a widely used plant for landscaping and for difficult growing sites, is highly susceptible to the pathogen *Ceratocystis fimbriata* f. sp. *platani* attacks. This work is focused on evaluating the effect of the presence of bacterial populations in explants showing an enhanced growth during *in vitro* cultivation. The explants were obtained from healthy plants of *P. acerifolia* W. growing in *C. fimbriata* infected areas. 30 bacteria strains have been isolated from *in vitro* explants and 18 were identified on the basis of their metabolic profile and molecular traits (*Paenibacillus aliginolyticus*, *Pigmentiphaga kulla*, *Blackwater bioreactans*, *Bradyrhizobium japonicum*).

Key words: *P. acerifolia*, *C. fimbriata*, beneficial bacteria, endophytes

Introduction

The canker stain caused by *C. fimbriata* f. sp. *platani* is one of the most serious diseases affecting *Platanus acerifolia*. The fungus was introduced in Europe in the middle of the last century from North America (Walter et al., 1952) and it shows an high level of homogeneity in the Italian population, with the same genotype throughout the Country (Santini & Capretti, 2000). Moreover, it is a pathogen with a broad range of host species and may cause severe losses and plant death. Since chemical control has been proved ineffective (Panconesi, 1981), an integrated strategy would help in the management of the disease. The genetic improvement of *Platanus* with the production of hybrids or the selection of resistant clones at present seems to offer a valuable strategy of countering the disease (Pilotti et al., 2002). Furthermore, an interesting approach for protecting planting material from early infections by the pathogen is the use of control measures, during plant propagation and/or at planting. The plant-associated microorganisms, endophytes in particular, were in the past always treated as problem causing contaminants of *in vitro* cultures (Leifert et al., 1994) and various procedures have been developed for their elimination as endogenous contaminants (Levin et al., 1979). Enhanced micropropagation capacity may be attributed to direct or indirect actions of microorganisms (Monler et al., 1998) with an improvement of plant performances under stress environments, where they can be employed as plant protecting or plant growth-promoting agents. (Novak, 1998; Monler et al., 1998). The objective of this research is the identification of bacteria isolated from *Platanus* explants showing an enhanced growth during *in vitro* cultivation. This is the first part of a project dealing with the improvement of *P. acerifolia in vitro* cultivation by the use of unknown and already known PGPR/BCA bacteria.
Materials and methods

In vitro culture condition for Platanus acerifolia f. sp. platani

Dormant shoots of *P. acerifolia* f. sp. *platani* were collected from healthy trees growing in *C. fimbriata* infected areas of Pisa (Italy). One noded cuttings were taken from actively growing shoots developed from axillary buds after about 1 month forcing in the growth chamber, at 25°C. Nodal cuttings were sterilized by sodium hypochlorite and planted in test tubes containing 10 ml of growth medium consisting of macroelements from DKW medium (Driver et al., 1984), microelements from MS medium (Murashige & Skoog, 1962), thiamin (1.0 mg/l), inositol (100 mg/l), sucrose (30 g/l), BA (0.4 mg/l), NAA (0.05 mg/l), agar Riedel (5 g/l) and pectin (2.5 g/l) (pH 5.5, 20 min at 121°C). The cultures were grown at 25°C under 16 h photoperiod provided by cool white fluorescent lamps (40± 5 µmol/m² s). The new shoots developed from axillary buds were used to start the shoot multiplication.

Bacteria isolation and identification

The bacteria were isolated from the micropropagation medium around the explant base. The basal portion from three different explants were transferred into 10 ml of 0.9% NaCl solution and rotary shaken for 30 min at maximum speed. Serial dilution were plated onto six different agar media: (DKW, MS, LB, YM, NfB, RMin). Isolates were purified by three cycles on the different media. The plates were incubated at 28°C for 3-5 days and colonies were selected on the basis of differences in colony morphology. The bacteria were typed by RAPD-PCR assay (to determine genetic similarity of genotypes) using the DAF₃ primer (5’-GGACCG AGCG-3’) as described by Corich et al. (2001) and identified by 16S rDNA sequence by using universal primers 27f and 1495r (Weisburg et al., 1991). Substrate utilization patterns of each isolate were obtained using Biolog GN MicroPlates (Biolog Inc., Hayward CS, USA) according to the manufacturer instructions. Metabolic profile were recorded manually after 24 h of incubation at 30°C.

Results and discussion

The aim of the present work is to contribute to the control of canker stain disease by finding endophytes effective as antagonist of *C. fimbriata* pathogens or able to improve *P. acerifolia* growth. 30 microorganisms have been collected from shoots of *P. acerifolia*.

Table 1. Cultivable bacteria from the basal portion of *P. acerifolia* explants on different media

<table>
<thead>
<tr>
<th>Medium</th>
<th>CFU/g dw</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>2.8±1.3 10⁹</td>
</tr>
<tr>
<td>YMA</td>
<td>4.0±1.9 10⁹</td>
</tr>
<tr>
<td>NfB</td>
<td>2.6±0.6 10⁹</td>
</tr>
<tr>
<td>RMin</td>
<td>2.4±1.1 10⁹</td>
</tr>
<tr>
<td>DKW</td>
<td>2.9±0.3 10⁹</td>
</tr>
<tr>
<td>MS</td>
<td>2.4±0.8 10⁹</td>
</tr>
</tbody>
</table>

After the isolation on different media (Table 1), a preliminary screening of the cultivable bacteria has been performed by substrate utilization patterns obtained using Biolog GN MicroPlates. Based on these analysis the isolated have been divided in 9 groups from which 18 isolated have been selected to be identified by 16S rDNA sequence. The results are reported in Table 2.
Table 2. 16S rDNA sequence of 18 bacteria isolated from *P. acerifolia* explants

<table>
<thead>
<tr>
<th>Bacterium species</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paenibacillus aliginolyticus</em></td>
<td>9</td>
</tr>
<tr>
<td><em>Pigmentiphaga kulla</em></td>
<td>6</td>
</tr>
<tr>
<td><em>Blackwater bioreactans</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Bradyrhizobium japonicum</em></td>
<td>1</td>
</tr>
</tbody>
</table>

The microorganisms isolated belong to species including strains able to stimulate plant growth or to perform as biocontrol agents or to perform nitrogen fixation. In order to clarify some biological properties, the 18 bacteria have been tested for indole-3-acetic acid production with Salkowsky method but they do not seem to be strong producers. In the same time *in vitro* test against *C. fimbriata* has been performed, but they do not seem to be active in the considered conditions. As showed in Table 2, 9 isolates belong to *P. aliginolyticus* that is the most represented specie in this work. *P. aliginolyticus* is an alginate degrading bacteria producing hydrolytic enzymes that may contribute to its antagonistic activity (Budi et al., 2000).

![Figure 1. RAPD profiles of the identified strains. 1-9 = *P. aliginolyticus*; 10,13-16,18 = *P. kulla*; 11-12 = *B. bioreactans*; 17 = *B. japonicum*. M = Low DNA molecular marker](image)

From the DNA RAPD-PCR analysis shown in Figure 1 it is not clear if the 9 isolates belong to the same strain or they are diverse, as they showed differences in substrate utilization by using Biolog GN MicroPlates (data not shown). The enhanced growth of the explants from which the bacterial endophytes isolated are origined may be due to the activity of a pool of microorganisms and the study of this aspect is in progress.

**Acknowledgements**

We gratefully acknowledge Prof. Marco Nuti for helpful comments and for critical reading of the manuscript. The research was supported by MIRAAF (Italian Ministry of Agriculture) and the Italian National Research Council (CNR).

**References**


Variability of the β-tubulin gene and intergenic spacer (IGS) region as an indicator for characterization of intraspecific variation in Japanese isolates of *Verticillium lecanii* (*Lecanicillium* spp.)

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**Abstract:** Recently, *Verticillium lecanii* was transferred and subdivided into *Lecanicillium lecanii*, *L. muscarium*, and *L. longisporum* according to morphological observations and PCR-RFLPs of ITS, mtDNA and β-tubulin (Zare & Gams, 2001). To clarify whether *V. lecanii* of mainly Japanese isolates were identified with *Lecanicillium* spp., DNA polymorphism in β-tubulin gene of *V. lecanii* from mainly Japanese isolates was analysed by PCR-RFLP. Those results showed that the examined isolates belonged to *L. muscarium* and *L. longisporum*, except for one isolate which alone did not belong to either *Lecanicillium* spp. Furthermore, PCR-RFLP in the intergenic spacer (IGS) region was also carried out to determine the genetic diversity. The isolates originated from whitefly except for one isolate consisted of one haplotype, whereas the isolates taken from aphids were included in the various IGS haplotypes.

**Key words:** *Verticillium lecanii*, *Lecanicillium*, β-tubulin gene, intergenic spacer, PCR-RFLP

**Introduction**

The deuteromycete *Verticillium lecanii* (Zimm.) Viegas has high pathogenicity and epizootic efficacy towards certain insects. Because of its entomopathogenic nature, certain isolates of *V. lecanii* have been developed as commercial microbial insecticides. Recently, it was suggested that a major part of the species formerly classified in *Verticillium* sect. *Prostrata*, especially *V. lecanii* and *V. psalliotae*, be transferred to *Lecanicillium*. Zare et al. (2000) used phylogenetic analysis of ITS sequences of nuclear rDNA to separate some species of *Verticillium* into at least four distinct clades. The distinctness of these clades was confirmed by Sung et al. (2001) by analyses of the combined SSU and LSU nuclear rDNA sequences in a wide array of taxa. Furthermore, *V. lecanii* was subdivided into three species and renamed *L. lecanii*, *L. muscarium*, and *L. longisporum* according to morphological observations and PCR-RFLPs of ITS, mtDNA, and β-tubulin gene (Zare & Gams, 2001). The present study clarifies whether *V. lecanii* of mainly Japanese isolates were identified with *Lecanicillium* spp. In addition, DNA polymorphism in the Bt-1 region of β-tubulin gene of *V. lecanii* isolates from different geographic origins and hosts were analysed using PCR-RFLP.

In addition to analysis of the Bt-1 region, the intergenic spacer (IGS) region of nuclear rDNA has been utilized extensively for inferring phylogeny among more closely related taxa. It has also been examined in the course of evolutionary and taxonomic studies of fungi. A
previous study demonstrated the extent of variability in these regions of *V. lecanii* (Sugimoto et al., 2003). The second purpose of present study was to inquire into the geographical distribution of variability and its relationships with *V. lecanii*-isolates’ origins and hosts.

**Materials and methods**

**Fungal isolates and DNA extraction**

This study used 66 isolates of *V. lecanii* from different geographic origins and hosts. Forty-one isolates were used in a previous study (Sugimoto et al., 2003). They were isolated mainly from Hokkaido (northern Japan), the U.S.A., and Europe. We newly isolated 4, 8, and 13 isolates from naturally infected whitefly or aphid corpses in greenhouses in Ibaraki, Chiba (middle of Japan) and Okinawa (south of Japan) prefecture, respectively. Okinawa is about 3,000 km from Hokkaido. After each fungal tissue for DNA extraction was grown in potato sucrose broth for 7 d at 25°C in darkness without shaking, it was harvested by filtering and rinsing with sterile distilled water. After excess liquid was removed, mycelia were ground to a fine powder under liquid nitrogen; DNA extraction was performed with the modified method of Li et al. (1994).

**PCR amplification of β-tubulin gene and the IGS region**

The PCR primers Bt1a & Bt1b (Glass & Donaldson, 1995) and CNL12 & CNS1 (Appel & Gordon, 1995) were used for amplification of the β-tubulin gene and the IGS region, respectively. DNA amplifications were carried out using a Takara Ex Taq kit (Takara Bio Inc., Shiga, Japan) with a GeneAmp PCR System 2700 thermal cycler (PerkinElmer Inc., Wellesley, MA, U.S.A.). Amplifications of β-tubulin gene and the IGS region were performed with the methods of Zare & Gams (2001) and Sugimoto et al. (2003), respectively.

**Restriction digestion, gel electrophoresis**

PCR products of β-tubulin region were digested individually with four different endonucleases (*Hae* III, *Alu* I, *Cfo* I, *Hinf* I), whereas *Sau*3A I, *Msp* I, *Hae* III, *Rsa* I, and *Pst* I were used for the restriction of the IGS gene region. Restriction fragments were size-fraction by electrophoresis through 1–2% agarose gels that were photographed under UV light after staining with ethidium bromide. Either presence or absence of bands was recorded in the IGS region.

**Results and discussion**

**PCR amplification and RFLPs of the β-tubulin gene region**

Of the 66 isolates, 65 showed a single amplified product of approximately 540bp in the Bt-1 region. All 65 isolates’ amplified 540bp showed similar fragment patterns as those digested with *Alu* I, *Cfo* I, and *Hinf* I, whereas two pattern were shown after digestion with *Hae* III. Thereby, these isolates were clearly distinguished two patterns (A and B type) (Figure 1). On the other hand, one isolate (ATCC58712) gave a 560 bp-amplification product; subsequent RFLP analyses of this product showed a unique fragment pattern. This haplotype was designated as C type. The isolates of type A corresponded with *Lecanicillium muscarium*; the isolates of type B were *L. longisporum* (Zare & Gams, 2001). The remaining isolates of type C did not belong to *Lecanicillium* spp. In addition, this study identified no isolate as *L. lecanii*. Identifying three haplotypes amongst the 66 isolates examined was associated with the previous study that was divided into three groups according to DNA polymorphisms of LSU mtDNA and Bt-2 region of β-tubulin gene, but not the ITS, IGS, SSU mtDNA, or histone 4 region. Particularly, many haplotypes of the ITS and IGS region were observed (Sugimoto et al., 2003). It is difficult to subdivide *V. lecanii* clearly into three species because
the classification according to the conidia morphology was vague in a previous study (Sugimoto et al., 2003). Further study and reconsideration are required.

Figure 1. RFLPs for *Verticillium lecanii* isolates in the β-tubulin gene region digested with *Hae* III, *Cfo* I, *Hinf* I, and *Alu* I. RFLP type is indicated by letters at top of the figure. The molecular weight marker is the 100 bp ladder.

**Table 1.** RFLP size (bp) of IGS haplotypes of *Verticillium lecanii*

<table>
<thead>
<tr>
<th>Haplotype</th>
<th><em>Msp</em> I</th>
<th><em>Hae</em> III</th>
<th><em>Rsa</em> I</th>
<th><em>Sau 3A1</em></th>
<th><em>Pst</em> I</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (21)</td>
<td>1200 530 380</td>
<td>390 300 280 240 180 170 120</td>
<td>490 430 380 250 160 120</td>
<td>1450 560 250 1500 910</td>
<td></td>
</tr>
<tr>
<td>II (2)</td>
<td>1350 530 380</td>
<td>390 300 280 240 180 170 120</td>
<td>490 430 380 250 160 120</td>
<td>1450 560 250 1500 910</td>
<td></td>
</tr>
<tr>
<td>III (1)</td>
<td>1150 530 380</td>
<td>360 300 280 180 170 150 120</td>
<td>490 430 380 250 160 120</td>
<td>1450 560 250 1450 910</td>
<td></td>
</tr>
<tr>
<td>IV (2)</td>
<td>1150 530 380</td>
<td>360 300 280 240 180 170 120</td>
<td>490 430 380 250 160 120</td>
<td>1450 560 250 1450 910</td>
<td></td>
</tr>
<tr>
<td>V (1)</td>
<td>1150 530 380</td>
<td>390 300 280 240 180 170 120</td>
<td>490 430 380 250 190 160 120</td>
<td>1450 560 250 1450 910</td>
<td></td>
</tr>
<tr>
<td>VI (2)</td>
<td>1150 530 380</td>
<td>390 300 280 180 170 120</td>
<td>490 430 380 250 190 160 120</td>
<td>1450 560 250 1450 910</td>
<td></td>
</tr>
<tr>
<td>VII (1)</td>
<td>1400 530 380</td>
<td>500 480 300 290 180 170 120</td>
<td>490 430 380 250 160 120</td>
<td>1500 560 250 1500 910</td>
<td></td>
</tr>
<tr>
<td>VIII (2)</td>
<td>1400 530 380</td>
<td>410 300 280 240 180 170 120</td>
<td>490 430 380 270 250 160 120</td>
<td>1500 560 250 1500 910</td>
<td></td>
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<tr>
<td>IX (10)</td>
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<td>400 300 290 280 240 180 170 120</td>
<td>490 430 380 250 180 120</td>
<td>1450 560 250 1500 910</td>
<td></td>
</tr>
<tr>
<td>X (1)</td>
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<td>500 300 290 240 180 170 120</td>
<td>490 430 380 250 160 120</td>
<td>1600 560 250 1590 910</td>
<td></td>
</tr>
<tr>
<td>XI (1)</td>
<td>1400 530 380</td>
<td>500 480 300 280 180 170 120</td>
<td>490 430 380 250 160 120</td>
<td>1600 560 250 1590 910</td>
<td></td>
</tr>
<tr>
<td>XII (1)</td>
<td>1400 530 380</td>
<td>500 300 290 180 170 120</td>
<td>490 430 380 250 160 120</td>
<td>1600 560 250 1590 910</td>
<td></td>
</tr>
<tr>
<td>XIII (2)</td>
<td>1450 530 380</td>
<td>500 300 290 280 180 170 120</td>
<td>490 430 380 250 160 120</td>
<td>1600 560 250 1590 910</td>
<td></td>
</tr>
<tr>
<td>XIV (6)</td>
<td>1450 530 380</td>
<td>550 300 280 240 180 170 120</td>
<td>490 430 380 250 220 160 120</td>
<td>1600 560 250 1590 910</td>
<td></td>
</tr>
<tr>
<td>XV (5)</td>
<td>1450 530 380</td>
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<td>490 430 380 250 220 160 120</td>
<td>1600 560 250 1590 910</td>
<td></td>
</tr>
<tr>
<td>XVI* (1)</td>
<td>1850 380</td>
<td>560 470 400 300 280 240 180 120</td>
<td>430 390 250 220 200 160 120 60</td>
<td>900 580 560 250 1600 660</td>
<td></td>
</tr>
<tr>
<td>XVII (2)</td>
<td>1650 380</td>
<td>490 400 280 230 120</td>
<td>430 390 250 220 200 160 120 60</td>
<td>900 580 560 250 1600 660</td>
<td></td>
</tr>
<tr>
<td>XVIII (1)</td>
<td>1850 380</td>
<td>480 340 280 240 210 120</td>
<td>430 390 330 250 220 200 160 120</td>
<td>880 580 560 250 2300</td>
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</tr>
<tr>
<td>XIX (3)</td>
<td>1350 460 380 370 200 190</td>
<td>600 420 400 340 220 110</td>
<td>1100 850 430 300 270 240 110</td>
<td>2000 800 560 250 4000</td>
<td></td>
</tr>
<tr>
<td>XX (1)</td>
<td>530 380 300 270 190</td>
<td>460 410 370 300 120 110</td>
<td>900 430 390</td>
<td>1100 280 250</td>
<td>990 840</td>
</tr>
<tr>
<td>XXI (1)</td>
<td>800 630 500 300 200 190</td>
<td>620 400 280 250 220 120</td>
<td>1500 430 270 220 110</td>
<td>1450 570 560 250</td>
<td>3000 2300</td>
</tr>
</tbody>
</table>

* Mycotal (Haplotype XVI), Vertalec (Haplotype XXI)

**Geographical distribution based on the IGS region**

The IGS amplified from the 25 newly isolated *V. lecanii* isolates was about 2.3 kb in length. Enzymatic digestions with *Sau*3A I, *Msp* I, *Hae* III, *Rsa* I, and *Pst* I showed several fragment patterns, reflecting intraspecific variation in this region. Four haplotypes were identified in the results: two haplotypes of those four were identical to a certain type, which had been described previously (Sugimoto et al., 2003). Other haplotypes were unique. The fingerprints generated from the restriction digestion of the IGS region demonstrated high levels of intraspecific variability, mainly with Japanese isolates used in this study. There were 21 different haplotypes amongst 66 isolates (Table 1).
Respective IGS regions of 16 out of 17 isolates which originated from whitefly in Japan consisted of one haplotype (Type I), but the isolated locations were a considerably long distance (from Hokkaido to Ibaraki and Chiba; c. 1,200 km). This result suggested that the original host could exert a selective pressure by favouring one genotype. The slight variety of DNA polymorphism may be attributable to the fact that whiteflies invaded Japan about 30 years ago; subsequently, they migrated and/or differentiated to various place of Japan in a few years (Yanagisawa, 1977). On the other hand, aphids had already invaded Japan 100 years or more ago. The examined isolates were taken from aphid corpses of three species, which were subjected to many environmental factors and were included in the various IGS haplotypes. Further studies are necessary to clarify whether these haplotypes were associated with host specificity and characteristics of populations in terms of evolutionary history, and maybe co-evolution.

References


Risk characterization of BCAs, side effects and environmental considerations
Compost as substrate for *Trichoderma*

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**Abstract:** The survival and establishment of a *Trichoderma atroviride* strain in three compost products were evaluated. Spent mushroom (*Agaricus bisporus*) compost at two different maturity levels (young and mature) and green compost from garden wastes were enriched with a sporal suspension of the antagonistic fungus. Peat moss and potting soil were used as controls. The population dynamic of *T. atroviride* was estimated at different times over a period of 140 d after enrichment. The potential toxic effect of enriched composts on the survival of the collembolan *Protaphorura armata* was also investigated. All compost products were suitable for the establishment of *T. atroviride*. Survival was the highest in mature spent mushroom compost. In particular, over the experimental period this compost product was able to sustain the population level of the antagonistic fungus obtained by enrichment. Moreover, mature spent mushroom compost promoted the highest level of rhizoplane colonisation ability of the antagonistic fungus. None of the tested products was toxic for *P. armata* specimens. Among microbial, physical and chemical parameters considered the survival of *T. atroviride* resulted only related to N form; the fungus seemed to survive better where NH₄⁺ was prevalent on NO₃⁻.

**Key words:** compost, *Trichoderma atroviride*, survival, Collembola, *Protaphorura armata*

**Introduction**

To successfully integrate the use of composts in crop production systems, composts could be enriched with selected strains of microbial antagonists to enhance the biocontrol effect of these organic products against plant diseases (Hoitink, 1990; Postma et al., 2003). However the success of antagonistic enrichment and durable survival of the introduced microorganism depends on the origin and quality of compost. In particular the nature of organic matter, maturity level and salinity are some of compost parameters that could strongly influence the activity of micro-organisms (Hoitink & Boehm, 1999). Into the soil the fortified compost interacts with components of the soil community, such as soil animals and very scarce are the studies on the interaction between fortified compost and soil animals.

We evaluated in this study the establishment and survival of a *Trichoderma atroviride* strain in different comports. We also try to find compost parameters that could provide the suitability of compost for enrichment. Moreover the effect of the enriched compost products on the survival of *Protaphorura armata*, a collembolan species used in ecotoxicological tests and well represented in soil community was studied.
Materials and methods

Organic products
The following compost products were used: young spent mushroom (*Agaricus bisporus*) compost taken after steaming at the end of production process (YSMC), mature spent mushroom compost taken 3 months after steaming (MSMC), and green compost derived from garden wastes taken one month after heat peaking (GC). Peat moss (PM) and potting soil (PS) were used as controls. Immediately before enrichment physical and chemical properties of the products were determined at the Laboratorio di Chimica del Suolo, University of Bologna. Population densities of indigenous aerobic bacteria, Actinomycetes, *Trichoderma* and total fungi were determined prior to the inoculation of the antagonistic fungus using a plate count technique on selective media (Potsma et al., 2003).

Test organisms
The benomyl-tolerant strain of *Trichoderma atroviride* Karsten 312 B2 (TA 312 B2) was used. The fungus is kept in the collection of the Dipartimento di Protezione e Valorizzazione Agroalimentare, University of Bologna. The fungus is characterised by a good antagonistic activity against some plant diseases. The acquisition of benomyl tolerance did not affect the fitness of the fungus (data not shown). The collembolans used belong to the species *Protaphorura armata* and were reared for several generations in the laboratory of Dipartimento di Biologia Animale, University of Modena and Reggio Emilia.

Bioassay
All products before enrichment were sieved trough 0.5 cm mesh. Each product contained into a black plastic bag (8 l) was enriched with a conidial suspension of TA 312 B2 to obtain a concentration of $8 \times 10^5$ conidia ml$^{-1}$ substrate. All bags were stored at 15-18°C in the dark. The content of each bag was periodically mixed and watered. All treatments were prepared in duplicate. The number of TA 312 B2 Colony Forming Units was determined by randomly sampling 10 g of each product per replicate at different times over a period of 140 d after enrichment. Samples were serially diluted and plated on TSM B10 medium (20 g agar, 3 g glucose, 1 g NH$_4$NO$_3$, 0.9 g K$_3$HPO$_4$, 0.2 g MgSO$_4$ 7H$_2$O, 0.03 g rose bengal, 50 mg streptomycin sulphate, 50 mg chloramphenicol, 2.5 g metalaxyl, 0.075 g pentachloronitro-benzene (99% a.i.), 10 mg benomyl/l deionised water).

The ability of TA 312 B2 to colonise the rhizoplane of wheat seedlings was assessed 100 d after enrichment. Four seedlings of 3 d old Creso durum wheat were transplanted into each 100 ml plastic pot containing each enriched product separately (30%), mixed with sand (66%), and perlite (4%) (v:v:v). Eight replicates were made for each treatment. All pots were maintained at 23°C, 16 h day light period, 70 % r.h. for 3 weeks. Thereafter seedlings were carefully collected, root systems excised and thoroughly washed in sterile water for 2 min. then plated on 90 mm Petri dishes containing TSM B10. After a week the total and colonised root length were measured by Axio Vision 2.05 image software. The root colonisation ability was expressed as the percentage of colonised root length out of the total root length.

The effect of enriched products on *P. armata* survival was tested by introducing 20 sexually mature animals of the same age, starved for 48 h, into each glass jar containing 30 ml of each enriched compost product separately. Jars containing potting soil were used as controls. All treatments were prepared in triplicate. The animals were extracted by flotation 2 months later and counted.
Results

Microbial counts, chemical and physical parameters are reported in tables 1 and 2. GC showed the highest numbers of aerobic bacteria, total fungi and indigenous *Trichoderma*, YSMC the highest number of Actinomycetes. Compost products had a similar pH that was higher than pH values of PM and PS. The electric conductivity (Ec) was higher in YSMC and MSMC than in other products. The NH$_4^+$-N content was higher in GC, and PM than in YSMC, MSMC and PS. The NO$_3^-$-N content was higher in PS and YSMC, than in other products. The NH$_4^+$-N/NO$_3^-$-N ratio was the highest in PM and very low in YSMC, MSMC and PS. The C/N ratio value was higher in PM than in other products. After incubation for 140 d the survival of the antagonistic fungus (tab. 3) was the highest in MSMC. In this compost the population level obtained by enrichment at the beginning of the experiment remained stable over the entire observation period. A negative relationship between TA 312 B2 CFU and NO$_3^-$-N was observed: $Y= -1093.9x+6377.2$ ($r^2=0.88$). A positive relationship between CFU and NH$_4^+$-N/NO$_3^-$-N was calculated: $Y=10^{-23}x^{30}$ ($r^2=0.76$). No correlation was found between numbers of TA 312 B2 and other parameters. Root colonisation ability of the antagonistic fungus was significantly the highest in MSMC (92%; a), intermediate in GC (43%; b) and PM (38%; b), and the lowest in PS (19%; c) and YSMC (16%; c) following LDS test at $P_{0.05}$. Data were arc sin transformed before ANOVA.

Table 1. Microbial counts of products before enrichment with antagonistic fungus

<table>
<thead>
<tr>
<th>Product</th>
<th>Aerobic bacteria</th>
<th>Actinomycetes</th>
<th>Trichoderma</th>
<th>Total fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young spent mushroom compost</td>
<td>8.7</td>
<td>7.7</td>
<td>4.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Mature spent mushroom compost</td>
<td>8.7</td>
<td>5.3</td>
<td>4.5</td>
<td>5.1</td>
</tr>
<tr>
<td>Green compost</td>
<td>9.1</td>
<td>7.0</td>
<td>5.4</td>
<td>6.9</td>
</tr>
<tr>
<td>Peat moss</td>
<td>5.5</td>
<td>4.7</td>
<td>2.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Potting soil</td>
<td>7.3</td>
<td>4.3</td>
<td>1.6</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Data are expressed in log$_{10}$cfu ml$^{-1}$

Table 2. Physical and chemical parameters of products before enrichment with antagonistic fungus

<table>
<thead>
<tr>
<th>Product</th>
<th>pH</th>
<th>Ec (mS cm$^{-1}$)</th>
<th>Ash (%)</th>
<th>Total N (g kg$^{-1}$)</th>
<th>NH$_4^+$-N (g kg$^{-1}$)</th>
<th>NO$_3^-$-N (g kg$^{-1}$)</th>
<th>NH$_4^+$-N/NO$_3^-$-N</th>
<th>Total C (g kg$^{-1}$)</th>
<th>C/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young spent mushroom compost</td>
<td>7.8</td>
<td>2.9</td>
<td>41</td>
<td>8.1</td>
<td>0.05</td>
<td>0.3</td>
<td>0.17</td>
<td>173</td>
<td>21.4</td>
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<tr>
<td>Mature spent mushroom compost</td>
<td>7.4</td>
<td>2.9</td>
<td>53</td>
<td>7.4</td>
<td>0.04</td>
<td>0.03</td>
<td>1.33</td>
<td>128</td>
<td>17.3</td>
</tr>
<tr>
<td>Green compost</td>
<td>7.7</td>
<td>1.3</td>
<td>40</td>
<td>18.3</td>
<td>0.3</td>
<td>0.05</td>
<td>6.0</td>
<td>295</td>
<td>16.1</td>
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<tr>
<td>Peat moss</td>
<td>5.6</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>5.6</td>
<td>0.2</td>
<td>0.02</td>
<td>10.0</td>
<td>345</td>
<td>61.6</td>
</tr>
<tr>
<td>Potting soil</td>
<td>5.0</td>
<td>1.5</td>
<td>6.7</td>
<td>13.0</td>
<td>0.07</td>
<td>0.6</td>
<td>0.12</td>
<td>318</td>
<td>25.5</td>
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</table>

None of the tested products was toxic for *P. armata* specimens. No differences were found among the numbers of alive adults collected from the enriched products and control jars. In addition to adults, also alive juveniles were found in all treatments.
Table 3. Numbers of *Trichoderma atroviride* 312 B2 after enrichment

<table>
<thead>
<tr>
<th>Product</th>
<th>2 d</th>
<th>6 d</th>
<th>16 d</th>
<th>24 d</th>
<th>48 d</th>
<th>90 d</th>
<th>140 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young spent mushroom compost</td>
<td>5.60</td>
<td>5.65</td>
<td>5.78</td>
<td>5.82</td>
<td>5.55</td>
<td>5.03</td>
<td>5.21</td>
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<tr>
<td>Mature spent mushroom compost</td>
<td>5.89</td>
<td>5.99</td>
<td>5.79</td>
<td>5.87</td>
<td>5.91</td>
<td>5.99</td>
<td>5.90</td>
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<tr>
<td>Green compost</td>
<td>6.06</td>
<td>5.77</td>
<td>5.84</td>
<td>5.58</td>
<td>5.68</td>
<td>5.80</td>
<td>5.32</td>
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<tr>
<td>Peat moss</td>
<td>5.78</td>
<td>5.74</td>
<td>5.97</td>
<td>5.92</td>
<td>5.88</td>
<td>5.38</td>
<td>5.49</td>
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<tr>
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<td>5.99</td>
<td>6.01</td>
<td>5.19</td>
<td>4.88</td>
<td>4.79</td>
<td>4.85</td>
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</tbody>
</table>

Data are expressed as log$_{10}$CFU ml$^{-1}$ product. Products LSD$_{0.05}$ = 0.10; sampling date LSD$_{0.05}$ = 0.11.

**Discussion**

The results of this study clearly indicate that *T. atroviride* 312 B2 survived at high levels in all compost products. The population level of the fungus was the highest in MSMC. This product sustained over the experimental period the sporal concentration optimal for a fortified compost (10$^5$ CFU ml$^{-1}$; Hoitink, 1990) obtained by enrichment at the beginning of the experiment. MSMC promoted also the highest level of rhizoplane colonisation ability of TA 312 B2, an important property for the success of the antagonistic fungus in the control of diseases caused by soil-borne fungi (Bailey & Lumsden, 1998). For these reasons MSMC resulted to be the most suitable substrate for the enrichment with the antagonistic fungus. From our data, TA 312 B2 seemed to survive better where ammonium was prevalent over nitrate. This is particular evident in light of data concerning mature and young spent mushroom compost. In fact, the latter characterised by a lower ammonium/nitrate ratio than the former, sustained lower survival and lower root colonisation ability of TA 312 B2. To be suitable for use into the soil, enriched compost must also be non toxic towards other components of the soil community; tests carried out with collembolans indicate that none of the tested products affected *P. armata* survival or blocked animal reproduction and development.

These results are a stimulus for a further step: evaluation of the suppressive effect of mature spent mushroom compost towards plant diseases caused by soil-borne fungi.

**References**


Replacement of copper fungicides in organic production of grapevine and apple in Europe (REPCO)

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The objective of our recently started project, partly funded by the European Commission (Framework 6; 501452), is to contribute to replace copper fungicides in organic agriculture by new measures for control of downy mildew (Plasmopara viticola) in grapevine and scab (Venturia inaequalis) in apple. Both major European organic crops strongly depend on copper fungicides. Permitted amounts will be reduced stepwise after 2006 (Council Regulation (EEC) 2092/91, Annex II) to avoid environmental risks. In European countries where copper fungicides are already out of use, production of organic apples suffers severe economical problems because of insufficient scab control. Potentiators of resistance, organically based fungicides and biocontrol agents will be screened and evaluated in grapevine and apple. The risk of pathogen evolution during use of novel control measures will be estimated to allow the development of sustainable strategies. Effects of crop management practices in organic agriculture on overwintering of V. inaequalis will be assessed. Novel disease control measures and knowledge will be integrated into organic management systems. 'Pipeline' products already under development elsewhere will be included and where necessary optimised in their use.
Evaluation of the effects of biocontrol agents (BCA) on the beneficial *Amblyseius andersoni* and the parasite *Tetranychus urticae* mites

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**Abstract**: The effect of commercial and experimental biocontrol preparations (AQ 10, Serenade, TRICHODEX and *Beauveria bassiana* strains B1 and B3) and of some fungicides (Ortiva, Tiovit jet) were tested in laboratory trials on the predatory mite *Amblyseius andersoni* Chant and on the spider mite *Tetranychus urticae* Koch. Direct and residual toxicity tests with females of the phytoseid showed that all the tested BCA based preparations and Ortiva are harmless (<30% of toxicity). Tiovit jet (31.61%) was slightly harmful. In contrast, the reference Pyrethrum showed a high toxicity level (100% mortality). Moreover, the tested BCAs did not interfere with the *T. urticae* spider mite populations, neither in term of adult survival nor in term of demographic parameters.

**Key words**: phytoseid mites, BCA, *Tetranychus urticae*, side-effect, laboratory, greenhouse

**Introduction**

The importance of *Amblyseius andersoni* (Chant) for the biological control of phytophagous mites in agroecosystem has been widely stated in the last thirty years (McMurtry & Van de Vrie, 1973; Baillod et al., 1982; Ivancich Gambero, 1985; Van de Vrie, 1985). Biological and ecological characteristics of this generalist predator, such as its high reproductive potential on various parasite mite species and pollens (Overmeer & Van Zon, 1983), make *A. andersoni* one of the most frequent dominant species on Italian crops.

In recent years, a greater attention to pesticides impact on environment has increased the interest on natural products, which are considered more acceptable than chemicals, because the former easily degrade in nature (Castagnoli et al., 2002). Herewith, we present laboratory investigations to test the side effects of eight biocontrol preparations administered by direct and residual contact on adult females of *A. andersoni* and evaluated the influence on the parasite mite population *Tetranychus urticae* Koch.

**Materials and methods**

**Mites rearing and origin**

One *A. andersoni* and one *T. urticae* strain were employed in the experiments. The first one was a wild strain collected during spring 2003 in an apple orchard while the second one was a two-year-old laboratory strain originated from bean cultivation. The phytoseid was mass-reared on artificial arena similar to that described by Angeli & Ioriatti (1994), fed with pollen of *Quercus* spp. (*Fagaceae*) and *Tetranychus urticae* Koch (*Acari: Tetranychidae*) and maintained in a climatic chamber at 25±1°C, 75±10 % RH and a photoperiod of 16L: 8D. The parasite mite *T. urticae* was mass-reared in greenhouse on bean plants.
**Test pesticides**

We used the five BCAs: AQ 10 (*Ampelomyces quisqualis*; 0.08 g/l), Serenade (*Bacillus subtilis*; 4 g/l), TRICHODEX (*Trichoderma harzianum* T39; 4 g/l), *Beauveria bassiana* B1 (5.27x10⁶ conidia/ml) and *Beauveria bassiana* B3 (6.55x10⁶ conidia/ml); Ortiva (a.i. azoxystrobin; 0.8 g/l), Tiovit jet (sulphur; 3 g/l). Commercial and experimental BCAs and fungicides were suspended in distilled water to produce concentrations equivalent to field application rates of 12 hl/hectare. Distilled water was used as control, Topas (a.i. penconazole; 0.4 g/l) as fungicide reference while the insecticide Piresan (pyrethrum; 1 ml/l) as toxic reference.

**Laboratory tests**

All trials were performed in climatic chambers at 25 ± 1°C, 75±10% RH and a photoperiod of 16L:8D. During the experiments on the side effect of BCAs and fungicides on *A. andersoni*, pollen of *Quercus ilex* and *T. urticae* mite was supplied as food twice a week (Angeli et al., 1996).

**Side effect of fungicides on *A. andersoni* – direct and residual toxicity on adult females**

Pairs of non-coeval females were placed on leaf disks of apple. Series of six females were treated under a Potter spray tower for 1 minute, with 8 ml of water solution of each chemical tested. The amount of fluid deposited on each disk was 1.7-1.8 mg/cm². Each BCA or fungicide was tested three times including a distilled water test as a control and a pyretroid as a reference. Mortality and female fecundity were assessed for seven days.

**Side effect of fungicides on *T. urticae* – direct and residual activity on adult**

Pairs of non-coeval adults were placed on leaf disks of apple; series of six adults were treated under a Potter spray tower using the same methodology described for the phytoseids. Each BCA product was tested three times. A distilled water test was included as untreated control and a pyretroid as a reference treatment. Survival and female fecundity was assessed for seven days.

**Analysis of data**

In the phytoseid trials (*A. andersoni* and *T. urticae*), the effect of the fungicides was expressed as: \[ E = 100\% - (100\% - M) \times R \], where \( E \) was the percentage of toxicity, \( M \) was the percentage of mortality calculated according to Abbott (1925), \( R1 \) was the fecundity [ratio between the number of eggs laid in the treated sample and in the control (Overmeer & Van Zoon, 1982)].

**Results**

**Direct and residual toxicity on *A. andersoni* females and effects on reproduction**

The effects of treatments on *A. andersoni* differed greatly from BCA treatments to the toxic reference Piresan (Table 1). On the female mortality, the BCAs were slightly toxic (Abbott mortality 0.9 \( \leq \) 10.5%) similar to Topas (2%), while it was highly toxic (100%) for Piresan.

The negative effects of BCA on the female-fecundity also differed significantly from BCA fungicide (4 \( \leq \) 23.6%), to the toxic reference (100%) (Table 2). Toxicity values calculated on the basis of mortality and fecundity showed that all the BCA are to consider harmless but for Tiovit jet that it resulted slightly toxic.

**Direct and residual activity on *T. urticae* and effects on reproduction**

Applying the Potter spray tower method, the effect of the BCAs on survival of *T. urticae* adults were similar to that of control (Figure 1). Furthermore, no significantly differences on the offspring were observed between the BCA fungicides and the references control and Topas.
Table 1. Toxicity effect (E) and Class of the fungicides on *A. andersoni* females

<table>
<thead>
<tr>
<th>Treatments</th>
<th>7-day- survival (%)</th>
<th>Oviposition (eggs/fem/day)</th>
<th>E (% toxicity)</th>
<th>Toxicity Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>1.06</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AQ 10</td>
<td>98.3</td>
<td>0.98</td>
<td>9.12</td>
<td>1</td>
</tr>
<tr>
<td>Serenade</td>
<td>92.1</td>
<td>0.82</td>
<td>28.76</td>
<td>1</td>
</tr>
<tr>
<td>TRICHODEX</td>
<td>99.1</td>
<td>0.96</td>
<td>9.82</td>
<td>1</td>
</tr>
<tr>
<td>Ortiva</td>
<td>94.2</td>
<td>0.88</td>
<td>21.8</td>
<td>1</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em> B1</td>
<td>96.8</td>
<td>1.02</td>
<td>6.85</td>
<td>1</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em> B3</td>
<td>97.9</td>
<td>0.95</td>
<td>12.26</td>
<td>1</td>
</tr>
<tr>
<td>Topas</td>
<td>98.0</td>
<td>0.98</td>
<td>9.4</td>
<td>1</td>
</tr>
<tr>
<td>Tiovit jet</td>
<td>89.5</td>
<td>0.81</td>
<td>31.61</td>
<td>2</td>
</tr>
<tr>
<td>Pyresan</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>4</td>
</tr>
</tbody>
</table>

The pesticides were classified according to 4 IOBC evaluation classes: Class 1 (harmless) = E < 30%; Class 2 (slightly harmful) = 30% < E < 80%; Class 3 (moderately harmful) = 80% < E < 99%; Class 4 (harmful) = E > 99%.

Figure 1. Effect of the BCA fungicides on the survival and the demographic parameters of *T. urticae*

**Discussion**

The selectivity levels of all the BCAs tested on the *A. andersoni* strain were high and similar to that of traditional selective fungicides (i.e. Topas); in contrast the reference insecticide pyrethrum (Pyresan) showed a high toxicity level. These results are similar comparing with that obtained in the field tests (Pertot et al., 2004). Moreover, the BCA fungicides tested did not interfere on the spider mite populations *T. urticae*, neither in term of adult survival nor in term of demographic parameters, similarly to that observed for Topas fungicide and untreated treatment tested. The laboratory trials have shown that all the BCAs and fungicides tested are harmless or slightly harmful on the strain of phytoseids used and do not interfere on the biological control of *Tetranichus urticae*. Regarding side effects BCAs can be safely included in IPM programmes.
Acknowledgements

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References


Environmental risk assessment of soil-applied fungal biological control agents with respect to European registration

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Abstract: As part of the EC-project RAFBCA (QLK1-CT-2001-01391), a three-year field trial with Beauveria brongniartii (Sacc.) Petch was carried out at the GLP/GEP-certified ATC-Agro Trial Center GmbH in Lower Austria to (i) study possible phytotoxic effects, (ii) determine if relevant fungal metabolites and residues enter the food chain and (iii) monitor the persistence and possible dispersal of the biocontrol agent (BCA). Furthermore, efficacy trials were conducted with Metarhizium anisopliae (Metschn.) Sorok. against grape phylloxera (Daktulosphaira vitifoliae (Fitch)) in vineyards with the additional goal to study non-target effects on soil fauna. The BCA densities in soils from both studies were compared with data collected on the diversity and abundance of common soil fungi. In order to complete the picture, the bacterial community was characterised by Community Level Physiological Profiling using the Biolog® system. No negative effects related to the application of the BCA could be detected in any of these studies. An overview of selected results is presented and guidelines for the judgement of risks posed by fungal biocontrol agents are discussed.

Key words: Beauveria brongniartii, biodiversity, field trials, Metarhizium anisopliae, risk assessment

Introduction

Entomopathogenic fungi are effective agents for the control of subterranean insect pests and provide farmers with a promising alternative to chemical pesticides. For instance, Beauveria brongniartii is successfully used as a biocontrol agent (BCA) against larvae of the common cockchafer (Melolontha melolontha L.) in Austria, Italy, Switzerland and France. However, the development of microorganisms as control agents also requires a responsible assessment of the risks that may be associated with their application. For instance, the fate of BCAs regarding their potential dispersal and establishment in the environment, the accumulation of metabolites in food, as well as non-target effects is an issue of concern (Strasser et al., 2000; van Lenteren et al., 2003). Field trials to assess risks associated with the application of fungal BCAs have been set up with B. brongniartii as part of the RAFBCA project (QLK1-CT-2001-01391) and with M. anisopliae as part of an efficacy study against grape phylloxera. These studies address important issues such as (i) possible phytotoxic effects caused by the BCA, (ii) the accumulation of fungal metabolites in food and feed, (iii) the potential dispersal of fungal inoculum, and (iv) the impacts which the introduction of large amounts of fungal entomopathogens may have on soil microbiota and soil fauna. The objective of our study was to generate data for an European registration of B. brongniartii and M. anisopliae and to provide a model approach for the in situ assessment of fungal biocontrol agents.
Material and methods

Field trial layouts
A full randomised block design (EPPO, 2004) with 3 treatments and 2 controls was used for the *B. brongniartii* risk assessment trial in Gerhaus, Lower Austria. A reference insecticide (Agritox®, Amtl. Pfl. Reg. Nr. 1797, Kwizda GmbH) and sterile barley kernels (33 kg/ha) served as controls, Melocont®-Pilzgerste (Amtl. Pfl. Reg. Nr. 2582, Kwizda GmbH) was applied in three different amounts (recommended rate of 50 kg/ha, the double and the fivefold of the recommended rate). Two of the four trial segments were re-applied with the same treatment one year after the 1st treatment to simulate a repeated application. Development of cultures was assessed following the recommendations of the European and Mediterranean Plant Protection Organization (EPPO, 2004), samples for residue studies were taken according to the highest available international standards. Kirchmair et al. (2004) used similar trial layouts for the efficacy evaluation of *M. anisopliae* against grape phylloxera, conducted in a vineyard near Geisenheim, Hessen, Germany.

Quantification of fungal BCAs in the soil
Soil samples from each layer were mixed, air-dried, and sieved through a 2 mm sieve. Ten gram sub-samples from each depth (three replicates) were added to 40 mL 0.1% (w/v) Tween80®, shaken at 150 rpm for 30 min, and then treated in an ultrasonic bath for 30 s. Agar plates selective for *Beauveria* (Strasser et al., 1996) and *Metarhizium* (medium from Strasser et al. (1996), supplemented with 22 g/L glucose monohydrate) were inoculated with 50 µL of these soil suspensions or dilutions thereof (four replicates per sub sample) and were then incubated for 14 days at 25°C and 60% relative humidity (RH). Colonies formed by *Beauveria* and *Metarhizium*, respectively, are given as cfu per gram soil dry weight.

Diversity of soil fungi
Potato-Dextrose-Agar plates supplemented with Streptomycin (100 mg/L), Tetracycline (50 mg/L) and Dichloran (2 mg/L as 0.2% w/v ethanolic solution) were inoculated with 50 µL of the extract obtained through the procedure described in the previous paragraph (four replicates per sub sample). The plates were incubated at 25°C and 60% RH. After one week the colonies were counted and assigned to the genera and taxonomical groups identified with microscopical methods.

Community level physiological profiling
Soil samples from each treatment (i.e. sterile barley, Agritox, three different amounts of Melocont®-Pilzgerste) were pooled. Bacterial cell extraction from the soil was performed following the procedure recommended by Insam & Goberna (2004). The obtained suspensions were transferred to ECOLOG®-plates which were incubated at 20°C / 60% RH for 7 days. Optical density was measured with a microtiter plate reader every 12 h at \(\lambda=592\) nm during incubation.

Data processing and statistical analyses
MS Excel 2000, SigmaPlot 8.0, SigmaStat 3.0, SPSS 11 and Statistica 6.1 were used for data processing and statistical testing. Multivariate datasets (fungal diversity, community level physiological profiling) were analysed with Principal Component Analysis (PCA), Discriminant Function Analysis (DFA), and MANOVA.

Results
Observed *Beauveria* densities in the soil correlated significantly with the applied amount of fungal barley and the number of treatments. In the plots applied once at the beginning of the
experiment, *Beauveria* densities peaked at above $10^5$ cfu per gram dry wt soil in the top soil (5-fold dose) and decreased to $10^3-10^4$ cfu/g dry wt soil 19 months after application. A single application of *Metarhizium* barley in German vineyards led to slightly higher BCA densities of $10^5-10^6$ cfu g$^{-1}$ dry wt soil.

Despite high BCA densities in soil no relevant differences in fungal diversity were estimated between treatments. The evaluated genera were evenly distributed within the data sets of both the *Metarhizium* trials in Germany and the *Beauveria* trials in Austria, with no differences detectable through PCA. When pooled datasets of the two trial sites in Austria and Germany were compared with multivariate techniques, the soils from the two sites were clearly distinguishable based on the evaluated soil fungi. Scatter plots obtained through PCA show distinct groups (Figure 1), which was confirmed by MANOVA analysis ($P<0.001$) and DFA, where 95% of cases could be correctly assigned to the sites.

Data from community level physiological profiling performed with soils from the *B. brongniartii* field trial resulted in distinct groups corresponding to treatments when analysed with DFA. A further analysis of these groups showed a significant correlation ($R^2 = 0.835$, $P<0.001$) between one DFA factor and the amount of barley kernels that had been applied to the respective trial plots (Figure 2).

![Figure 1](image1.png)  
**Figure 1.** Scatter plot calculated by PCA from fungal diversity data at the two trial locations.

![Figure 2](image2.png)  
**Figure 2.** Correlation (with 95% conf. limits) of DFA output with amount of applied barley.

**Discussion**

None of the data from these *in situ* studies indicates environmental risks posed by the application of either *B. brongniartii* or *M. anisopliae* to the soil.

The abundance of different species of Acari, Collembola or Lumbricida was not affected by applied *Metarhizium* in vineyards (Kirchmair et al., 2004). Similar observations have been made by Dromph (2001) and Hozzank et al. (2003) for *B. brongniartii* and *M. anisopliae*, respectively. None of the cultures grown after the application of *B. brongniartii* showed any phytotoxic reactions or yield reductions compared to the controls (Längle et al., 2004), and no observations of phytotoxic effects have been described in the field after soil application of *Metarhizium*. Despite high BCA densities in the soil – mostly above recommended control thresholds of $5 \times 10^3$ cfu/g dry wt soil (unpublished data) – neither the indigenous soil fungi
nor the bacterial community were negatively affected by the BCA itself. Observed differences in the bacterial community were clearly correlated to the amount of carrier material, i.e. food grade barley, and suggest a fertilization effect. This observation, as well as the clear differentiation of the German and Austrian sites on the basis of fungal diversity, show the high discriminative power of the applied techniques and support the assumption that the soil biota is not affected by the application of fungal BCAs.

It could be said that, as a consequence of available risk assessment data, fungal entomopathogens will be listed in Annex I of Council Directive 91/414/EEC in the near future. However, obstacles still have to be overcome, because every member state is authorized to assess BCAs by taking into account local climate, cropping pattern and diet. From today’s perspective this will delay the use of reliable control agents.

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References


Risk characterization of a potential biocontrol agent: receptor identification, risk assessment and management

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Abstract: Micro-organisms to be used as biological control agent have to follow the registration procedures of a pesticide, which is a cumbersome and costly undertaking. This is a main constrain in developing a biocontrol agents (BCAs) up to marketability, moreover as the market is mostly restricted to organic farming. By applying the same procedure for registration as for chemical pesticides, relevant factors such as risk differences at any particular stage of the use between a naturally occurring micro-organism and a new, in nature unknown synthetic molecule are not considered. If a biological control agent is to be isolated, studied, handled, multiplied in order to be finally released into the environment, scientists and regulatory authorities need to weigh the benefits of pest or disease control against the risks posed to the researchers, workers as well as no target plants or animals. Although potential risks to non-target species are recognized and attempts are being made to determine the nature of this, a documentation of such side effects has generally been poor and some reports even disputed. Future biological control programmes will need to assess carefully the potential impact of introduced agents on non-target species and on ecosystems as a whole. In this paper we analyse which techniques have and can be considered for a BCA-monitoring as a first step for a wide approach to characterize human and environmental risks of employing micro-organisms as BCA.

Key words: risk assessment, monitoring, BCA

Introduction

Two of the main constrains in biocontrol agents (BCAs) development as pesticides are the expensive requirements for their registration and their small market dimension, mainly restricted to organic agriculture (REACH – New chemicals registration). In the EU countries there are common principles for the evaluation of plant protection products, regulated by the Council Directive 91/414/EEC. For BCA registration as plant protection product, companies must follow a complex protocol similar to the protocol required for chemicals. The BCA is actually considered as a chemical molecule. This is a prudential way of acting, due to the lack of specific knowledge on BCA risk characterization and management. For some microorganisms (MOs) with proven lack of toxicity and with a potential role as biocontrol agents, some of the requested information in the dossier could be unnecessary. A critical evaluation of the literature could be the starting point for the definition of new guidelines and hopefully in the simplification of the rules for the registration of a BCA as a pesticide in Europe. Bibliography on BCA risk assessment can be roughly divided into three main areas: human health, ecotoxicology and environmental fate. In this paper we will focus the environmental fate of released BCAs. We aim to list the most commonly used methods allowing the assessment of the stability of the introduced BCA into a new environment in the phases of deposition, reproduction, colonization and persistence (monitoring).
Monitoring tools and methods

In the literature the reader is often confused by the term “risk assessment”, which is erroneously used as a synonym of “monitoring”. Monitoring however implies only a regular check on how the BCA population changes or progresses without attributing a risk factor to the data. A universal method for monitoring cannot be identified: in each situation it has to be adapted to the specific questions and conditions. Monitoring studies for detection and identification of spatio-temporal environmental fate of MOs rely on one or more groups of techniques. The choice of the technique depends on: i) the objective of the detection or identification process, ii) the characteristics of the microorganism and iii) the environment in which that microorganism has to be detected.

Essentially five groups of environments can be distinguished: air, sediment, soil, water, and living organisms (plants, animals, MOs). MOs can be collected from the different environments with specific tools. Sedimentation, centrifugation, impaction, filtration, impingement, and electrostatic methods allow the MOs to be collected from air samples (Valadares et al., 2001; Williams et al., 2001).

MOs can be extracted from aquatic environments simply by taking a sample of the water (Nasser & Oman, 1998; Winkler et al., 1995). Further centrifugation can be used to extract MOs from water samples employing or not a density gradient (Quintero-Betancourt et al., 2002). MOs can be extracted from soil by mixing a sample of soil in a buffered solution. Extraction of MOs from soil can be improved by sonicating the soil in buffer (Elsas et al., 1998, DeFlaun et al., 2001). Due to the similar physical characteristics between sediment and soil, the same techniques can be used to extract MOs from both environments (Khoshmanesh et al., 2001). Sediment is predominantly an anaerobic environment. There are two main types of device for sampling sediment: grab and core samplers.

Within the collected sample, the released BCA can successively be detected. A wide spectrum of MO detection methods can be considered (Elsas et al., 1998): culture based techniques (Bölter et al., 2002), Fourier transform infrared spectroscopy (Mariey et al., 2001), biolog assays (O’Connell et al., 2000), fatty acid analysis (Zelles, 1999), bioluminescence (Ramos et al., 2000), microscope-based techniques (brightfield-, phase-contrast-, epifluorescence-, immunofluorescence-, electron-microscopy, Verran et al., 2002), sampling strips (Urzi & De Leo, 2001), flow cytometry (Davey et al., 1999), fluorescence in-situ hybridization (FISH, Aulenta et al., 2004), DNA microarrays (Denef et al., 2003), colorimetric-based techniques (enzyme linked immunosorbannt assay, Van Weemen & Schuurs, 1971), reported gene based techniques (Germida et al., 1998) and molecular-based techniques as nucleic acid probes (Esiobu et al., 2004; Blomster et al., 2000), gene sequencing (Patel, 2001) or PCR (Heuser & Zimmer, 2002; Woo et al., 1999). For monitoring studies, only culture based techniques and molecular techniques or a combination of them satisfied the criteria of high specificity, repeatability, reliability, amenability to automation and low quantities of material required.

Due to their simplicity and low cost, culture-based techniques are the most common method for detecting and identifying MOs extracted from the environment. The main constrain in using culture-based techniques, is that, to be detected or identified, the microorganism must be in vitro cultivable. Less than ten percent of the all MOs in the environment are reported to be cultivable (Torsvik et al., 1994). To ensure a correct identification of the MO, molecular based techniques can further be coupled.

Molecular-based techniques involve the detection and/or identification of a specific component of the microbial cell. In general molecular techniques usually refer to the analysis or detection of a specific gene or nucleic acid sequence. Analysis, identification of MO-
specific nucleic acid sequences often requires the DNA to be extracted from the microorganism prior to analysis (Möller et al., 1992; Malik et al., 1994; Dilworth & Frey, 2000; Robe et al., 2003). To be successively amplified DNA must be imperatively pure. Several methods are available for removing PCR inhibitors (mainly polysaccharides and phenols) but none can be used for all protocols (Moreira, 1998; Koonjul et al., 1999; Perch-Nielsen et al., 2003).

Sequence Tagged Site (STS) molecular markers must be used. They must be able to amplify exclusively a genomic sequence belonging to the BCA strain released to the environment (Rafalski et al., 1996). The most important of these is the microsatellite or simple sequence repeat (SSR) marker (Zane et al., 2002) and SCAR markers (sequence characterized amplified regions, Gu et al., 1995). The presence or absence of amplification products is an evidence of qualitative presence or absence of the BCA in the sample collected.

**Sampling procedures**
Samples must be collected according to a suited sampling strategy. As dispersal/spread are concepts including both spatial as temporal variables, a sampling strategy should include both spatial and temporal dimensions (Zerbath et al., 2002). Four basic types of methodologies are most commonly used for conducting samples; these are simple random, stratified, cluster, and systematic sampling. Simple random sampling provides the base from which the other more complex sampling methodologies are derived (Buckland et al., 1993).

*Simple Random Sampling.* To conduct a simple random sample, the researcher must first prepare an exhaustive list (sampling frame) of all members of the population of interest. From this list, the sample is drawn so that each item has an equal chance of being drawn during each selection round.

*Stratified Random Sampling.* Stratified random sampling involves categorizing the members of the population into mutually exclusive and collectively exhaustive groups (strata). An independent simple random sample is then drawn from each group. The basis for effective stratification may be (micro)topography, type of vegetation, soil type, the target and the non-targets of interest.

*Cluster Sampling.* Cluster sampling is similar to stratified sampling because the population to be sampled is subdivided into mutually exclusive groups. After the clusters are established, a simple random sample of the clusters is drawn and the members of the chosen clusters are sampled.

*Systematic Sampling.* Systematic sampling, a form of one-stage cluster sampling, is often used in place of simple random sampling. In systematic sampling, the researcher selects every $n^{th}$ member after randomly selecting the first through $n^{th}$ element as the starting point.

As in a biocontrol strategy we distinguish among a target organism (or a tissue or an organ of it) and numerous non-target organisms, which represent mutually exclusive groups, the stratified sampling strategy and the cluster sampling strategy are suited (Godoy & Jordano, 2001).

An initial assessment must be performed before treatment of the MO within and outside the experimental plot and in surrounding regions on possible hosts. This sampling will exclude exo-contamination from the surrounding regions (immigrations) and set the level of BCA before the initial treatment (Broggini et al., 2004). Sampling immediately after treatment with MO provides the most reliable information in predicting the effect of MO dispersal/spread on crop response. Further samplings will provide a more precise dispersion dynamic of the BCA and the hosts where the BCA survives on. Quality control samples (blank samples) must be collected in order to exclude contamination during the sampling procedure, the transport to the lab, the sample handling and the performance of the methodology.
Since biocontrol can be applied in several environments and involves different host/pathogen/BCA biological systems, it is not possible to define precise rules. The number of samplings, time lag between samplings, the type of non-target strata, the number of samples per receptor (stratum), as well as many other specific factors will be undertaken to the personal judgment of the researchers and argued from the literature available. Collecting samples from every receptor may be technically very complicated, time consuming, and the number of the samples collected (including repetitions) may easily exceed the technical possibility of analyzing them in a reasonable time. In order to be realistic, the sampling can be initially performed by collecting a relative low number of samples from strata of major interest. Then further sampling can be performed according to the preliminary results.

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References


Safety in research: biocontrol agents and semiochemicals
risk management in the laboratory

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Abstract: Occupational safety and health in work environments of research and development of semiochemical and microbial biocontrol agents is currently not regulated by specific standards or directives. Workers in BCAs R&D laboratories are exposed to risks that can be minimised if appropriate procedures are applied. The aim of this study was to define simple guidelines for projecting new laboratories specifically dealing with BCAs, which will allow avoiding time and money consuming procedures.

Key words: risk management, biosafety, microbiological laboratory

Occupational safety and health in work environments of research and development (R&D) of semiochemical and microbial biocontrol agents (BCAs) is currently not regulated by specific standards or directives. Currently in BCAs and semiochemicals R&D the Good Laboratory Practice (GLP) for standard microbiology laboratories are used, however BCAs may pose different problems or allow a partial simplification in some laboratory procedures. In the USA, the Occupational Safety and Health Administration (OSHA) proposes to adopt the standards, which address indoor air quality in indoor work environments. OSHA gives also the rules that justify the use of respirators to prevent the inhalation of harmful airborne contaminants that are alive or were released from a living organism and that cause a range of diseases. In the EU, the European Agency for Safety and Health at Work acts as a catalyst for developing, collecting, analyzing and disseminating information that improves the state of occupational safety and health, but at the moment there are no specific directives on this subject.

Exposure to fungi and moulds can cause symptoms such as nasal stuffiness, eye irritation, or wheezing. People with allergies to moulds, may have severe reactions. Persons with a reduced immuno-system activity may be at risk to develop mycoses. Therefore isolating and managing unidentified microorganisms could be harmful if precise procedures are not followed. Some episodes of allergies have been reported among people involved in rearing insects for experimental trials.

The strategy for minimizing the occupational exposure of laboratory workers and the surrounding environment to infectious agents is actually based on the concept of micro-organism containment, which includes physical factors (e.g., facility design and safety equipment), standard microbiological practices, and administrative controls.

The aim of this work was to characterize the risk conditions related to dealing with unidentified micro-organisms and to rearing insects in the lab, by analysing and reviewing the job processes. In particular we dealt with the procedures required in research and develop-
ment of BCAs and semiochemicals. In this study the method of "congruenze organizzative" developed by Maggi (1986) has been used. The resulting document was used as guideline during projecting a new building hosting BCA-research.

The risk related to manipulating unidentified microorganisms, mass production of potential BCAs and rearing insects was characterized. Potential risky situations (separately classifying risks and harms) were identified. Using a draft project of the building, the potential risk situations were analyzed following the precise pathway, related to each procedure that can be harmful for workers. Risk of accident, risk related to manipulation chemicals and unknown substances or biological agents were identified. The list of potential human pathogens which can be involved in specific laboratory infections reported by Sewell (1995) was used.

Identified risk situations were eliminated through specific modification in the preliminary project or by proposing alternative pathways and novel solutions. Precise protocols were formulated for each procedure. Advices for individual protection were included whenever necessary. Isolation and identification of potential BCA is the step, which requires safety equipment such as biological safety cabinets (BSCs of type I or II), safety centrifuge containers, and personal protective equipment (e.g., gloves, masks, face shields and glasses, coats, and gowns). Isolating micro-organisms (MOs) using some precautions (e.g. not isolating from animal derived materials or from human, discarding MOs growing above 36°C, discarding MOs with colony growth similar to the listed human pathogens or allergens or toxin producing ones, using isolation media which do not contain animal derived proteins, etc.) will drastically reduce the risk of isolating harmful organisms. Appropriate air management in the different areas of the laboratory and basic lab safety rules (HHS publication U.S. 93-8395, 1993; Richmond & McKinney, 1999) are considered sufficient for all the other procedures in BCAs R&D.

Guidelines for planning and managing a laboratory that deals with research and development of BCAs and semiochemicals were produced and is available at the web site “http://www.safecrop.org/services/index.html”. These guidelines can also be a valuable tool for increasing safety in labs which already working in this field of research.

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References


Biocontrol on seeds
**Trichoderma** applications on sugar beet leaves reduce lesion size and sporulation of *Cercospora beticola* and increase sucrose yield

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**Abstract:** Cercospora leaf spot, caused by *Cercospora beticola* Sacc. is considered one of the main sugar beet diseases in southern Europe, negatively affecting root yield and sucrose content. Genetic resistance in sugar beet is only partial and the control of the disease is mainly achieved through repeated fungicide applications. Today, there is growing interest for alternative ways of control to be used in integrated or biological crop systems. This study reports the evaluation of some *Trichoderma* isolates as biocontrol agents of *C. beticola*. Some preliminary laboratory and greenhouse screenings and two-year open field trials led to the selection of two *Trichoderma* isolates that were tested again in 2003 under natural *C. beticola* inoculum in northern Italy. The climatic conditions delayed the appearance of the pathogen, and the disease severity was below the usual level for this environment. Repeated foliar applications of the isolates did not reduce the disease incidence (0 to 9 KWS scale). Nevertheless, one isolate led to a sucrose yield not statistically different from that of the treated control, represented by two traditional sprayings of fungicide, and significantly higher than the untreated control. It also significantly reduced *C. beticola* sporulation per unit of necrotic area in a way similar to the fungicide. Both isolates and the fungicide reduced the lesion size by up to 50%. Root yield was not affected by any treatment. The study confirms the effectiveness of *Trichoderma* foliar applications for reducing cercospora leaf spot of sugar beet, with a performance not different from two fungicide applications, at low disease pressure conditions.

**Key words:** *Trichoderma, Cercospora beticola*, sugar beet, integrated control

**Introduction**

Cercospora leaf spot, caused by *Cercospora beticola* Sacc. (Fungi Imperfecti, order Moniliiales, family Dematiaceae), is the most important sugar beet disease in southern Europe, causing considerable reductions in root yield and sucrose content. Control of the disease is generally performed by the use of partially resistant genotypes and repeated fungicide applications. Genetic resistance is polygenic and of the “rate reducing” type and cannot guarantee full protection (Rossi et al., 1999). Recently, growing interest has arisen for alternative ways of control, due to the necessity of reducing chemical pollution of the environment. A promising way of achieving an acceptable plant protection level is to integrate the use of genetic resistance with a biocontrol agent while reducing the number of fungicide applications.

*Trichoderma* spp. are known as effective biocontrol agents of a number of pathogens, due to mechanisms of action, such as competition, antibiosis, mycoparasitism, induction of defence response and other adjunct mechanisms, such as growth promotion, which could positively affect plant response to infections (Tronsmo & Hjeljord, 1998). *Trichoderma* isolates have been used in a wide number of crops against soil-borne, and seed-borne diseases and storage rots. Some positive results have also been reported for foliar diseases, as for
Botrytis cinerea (Elad et al., 1993). Having obtained encouraging information about the effectiveness of selected Trichoderma strains against C. beticola in sugar beet, we carried on studying their possible integration in protection programs against the disease (Galletti et al., 2002; Cerato et al., 2004).

Materials and methods

Preliminary laboratory, greenhouse and field trials on sugar beet led to the selection of two Trichoderma strains, Ba12/86 and Ba9/86 differing for antagonistic characteristics and field response and belonging to different species, T. longibrachiatum Rifai and T. hamatum (Bonorden) Bainier, respectively (Cerato et al., 2004). They were all isolated from sugar beet soil from Battipaglia (Salerno), in southern Italy, in 1986. These isolates were tested in 2003 in a field trial at Rovigo, in northern Italy, under natural C. beticola inoculum, on the partially resistant cultivar ‘Monodoro’, in a randomised block design with six replicates and 9 m² plots.

The isolates were cultured on 200 ml PDB for 10 days at 28°C, in the dark, then the homogenate was diluted with water to 1 L and sprayed on the sugar beet leaves of each plot at 15 day intervals, between June 24th and August 19th. The homogenate applications followed a single preventive treatment, on June 10th, with 25% difenoconazole (Score 25 EC, Syngenta, 250 ml/ha). Trichoderma based treatments were compared to an untreated control (water) and to a conventional control, 3% cyproconazole and 9% fentin acetate (Alto BS, Syngenta, 2 Kg/ha), applied on July 8th and August 5th. In addition, Ba12/86 was tested in a second way, bringing forward the first application to the middle of May and then continuing the treatments till August 19th.

On August 28th, 20 leaves (5x4 blocks) for each treatment were randomly sampled, 10 disks with one spot each were cut from each leaf, put in a micro-centrifuge tube in 400 µL water and shaken for 2 min to release spores. The leaf disks were then computer scanned and the necrotic area of each spot was calculated using the software program ‘ImageJ 1.23’ (http://rsb.info.nih.gov/ij). Spore production was calculated (mean of 8 counts per sample) using a Burker hemocytometer and the number of spores per mm² of necrotic area was calculated for each leaf.

Disease incidence was evaluated on August 29th by a KWS scale-based score (0 = 0% of leaf necrotic area, 9 = 100% of leaf necrotic area). Roots were harvested on September 10th and root and sugar yields were evaluated. Data were submitted to analysis of variance. Means were separated by the LSD test at $P \leq 0.05$ significance level, by MSTATC 2.1 microcomputer statistical program.

Results and discussion

An extremely hot and dry summer delayed the appearance of the disease. The first spots of the pathogen were observed at the beginning of July, and for this reason early difenoconazole application may have been not so effective, in spite of its systemic action. The disease severity was in fact below the usual level in the same period for this environment.

The different treatments did not reduce the disease incidence, which at the end of August was scored at between 7 and 7.3 (KWS scale) for all treatments, excluding the treated control which was significantly less damaged (Table 1). Root yield was not significantly affected by any treatment, even if absolute values ranged from 80 t/ha for untreated control, to 86 t/ha for treated control (Table 1).
Table 1. Disease incidence, root and sugar yields after *Trichoderma* treatments in the field trial at Rovigo (Italy), in 2003

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Disease incidence (KWS scale 0-9)</th>
<th>Root yield (t/ha)</th>
<th>Sugar conc. (%)</th>
<th>Sugar yield (t/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated control</td>
<td>5.8 a*</td>
<td>86.53 ns</td>
<td>15.13 ab</td>
<td>13.01 a</td>
</tr>
<tr>
<td>Untreated control</td>
<td>7.2 b</td>
<td>80.25 ns</td>
<td>14.56 bcd</td>
<td>11.46 cd</td>
</tr>
<tr>
<td>Difenocon. + Ba9/86</td>
<td>7.3 b</td>
<td>84.62 ns</td>
<td>14.75 abc</td>
<td>12.46 ab</td>
</tr>
<tr>
<td>Difenocon. + Ba12/86</td>
<td>7.0 b</td>
<td>81.78 ns</td>
<td>14.74 abc</td>
<td>12.02 bc</td>
</tr>
<tr>
<td>Anticipated Ba12/86</td>
<td>7.2 b</td>
<td>81.54 ns</td>
<td>14.33 cd</td>
<td>11.65 bcd</td>
</tr>
</tbody>
</table>

*Values followed by the same letter are not different for P≤0.05 (l.s.d. test); ns: not significant

Sugar content was generally high, about 14-15%, due to the long dry season, but significant differences were not found between the controls (Table 1). Nevertheless, Ba9/86 based treatment led to a sugar yield not statistically different from that of the treated control, and significantly higher than the untreated one (tab.1). This effect is probably linked to the specific biological agent, or at least to a synergy with the fungicide, since the sugar yield obtained with the other isolate (Ba12/86), following difenoconazole application, did not differ significantly from that of the untreated control (Table 1).

Ba9/86 also significantly reduced *C. beticola* sporulation per unit of necrotic area, measured at the end of August, in a way similar to the fungicide treated control (Table 2). On the contrary, Ba12/86 did not affect spore production when it followed difenoconazole application nor did it increase it in anticipated and prolonged applications. These effects on sporulation confirm what was found for the two isolates in a previous field trial under naturally severe inoculum pressure (Cerato et al., 2004).

Table 2. *C. beticola* lesion size on the leaves and spore production per unit of necrotic area after the different treatments in the field trial at Rovigo (Italy), end of August 2003

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Mean lesion size (mm²)</th>
<th>Spore production (no/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated control</td>
<td>5.54 b*</td>
<td>177 d</td>
</tr>
<tr>
<td>Untreated control</td>
<td>7.58 a</td>
<td>446 b</td>
</tr>
<tr>
<td>Difenoconazole + Ba9/86</td>
<td>4.76 b</td>
<td>184 d</td>
</tr>
<tr>
<td>Difenoconazole + Ba12/86</td>
<td>4.52 b</td>
<td>429 bc</td>
</tr>
<tr>
<td>Anticipated Ba12/86</td>
<td>4.36 b</td>
<td>669 a</td>
</tr>
</tbody>
</table>

*Values followed by the same letter are not different for P≤0.05 (LSD test)

Both isolates and fungicide (treated control) reduced mean lesion dimensions, unlike the untreated control that showed lesions of almost double size, indicating a slow-down of the pathogen development inside the treated tissues. This effect seems not to be due to difenoconazole, since it was also observed with Ba12/86 when applied alone (Table 2). It could be due, instead, to the direct competition between *Trichoderma* and *C. beticola*, in planta, especially for Ba9/86, which in vitro really showed a stronger inhibition effect (over 60%) on *C. beticola* mycelial growth, than Ba12/86 (about 20%). The mechanism at the base
of Ba12/86 action should, instead, be more indirect and due to induction of defence response, as reported in a study on the biochemical response of different sugar beet genotypes artificially inoculated with *C. beticola* and treated by Ba12/86, in greenhouse (Roberti et al., 2003). Nevertheless, in this field trial, this strain lacked effectiveness, not confirming the positive results obtained during the previous two years, maybe due to a greater sensitiveness than Ba9/86 to the particularly hot and dry season (Galletti et al., 2002; Cerato et al., 2004).

In conclusion, even if there was not a complete correspondence between the different years, due to very different climatic conditions and disease incidence, the study confirms some effectiveness of an integrated protection system based on *Trichoderma* foliar applications, following a single fungicide pre-treatment, for reducing cercospora leaf spot of sugar beet. This integrated program led to a performance not different from a conventional chemical protection by two fungicide applications, at low disease pressure conditions. Further studies are needed to confirm the effectiveness of this approach under different natural environments.

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Screening of microorganisms and other alternative seed treatments for activity against seed-borne pathogens of cereals

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Historically, seed-borne pathogens have been one of the major obstacles in cereal production. In conventional agriculture where highly effective chemical seed treatments are regularly used, seed-borne pathogens are nowadays considered only a minor problem. However, in organic farming seed-borne pathogens are apparently becoming increasingly important. The main reason is the lack of effective methods of seed treatment allowed to be used in organic farming. We performed a screening under controlled conditions for activity against common bunt (*Tilletia caries*) of wheat with laboratory preparations of microorganisms belonging to different taxa (*Trichoderma*, streptomycetes and other bacteria), formulated microbial products, plant extracts and resistance inducing agents. Treatments with activity were observed in each of these groups, except in the group of resistance inducing agents. When effective treatments were repeatedly tested, a large variability was observed between the experiments. This was especially true in case of the bacteria, but less pronounced for the tested isolates of *Trichoderma* and *Streptomyces*. The most active treatments were included in a field trial and further evaluated in greenhouse tests for activity against other important cereal seed-borne pathogens (leaf stripe of barley, *Helminthosporium gramineum*; net blotch, *Drechslera teres*; *Fusarium*-seedling blight). The results will be presented and discussed.
Influence of wheat seed treatment with Clonostachys rosea on the expression of PR proteins

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Abstract: The possible involvement of PR1, PR3 (chitinase), PR4 (wheatwins), PR5 (osmotin) and PR9 (peroxidase) proteins and genes in wheat systemic resistance induction by the mycoparasite Clonostachys rosea 47 (CR47) has been investigated. Wheat seeds cv. Creso were coated with CR47 and 6 days old coleoptiles were inoculated with Fusarium culmorum (FC). Seedlings and roots were harvested 7, 8, 9 and 10 days after the treatment and either RNA or total proteins were extracted. Peroxidase activity determined after isoelectrofocusing (IEF), 5-8 pH range, increased with the time. A peroxidase isoform (pI close to 6.3) was induced in coleoptiles by FC inoculation (I) and its expression was enhanced in CR47-treated seeds inoculated with FC (T+I) 10 days after treatment. Moreover, this isoform was always reduced by CR47 treatment alone. Chitinase activity in coleoptiles was stimulated by FC at 24 and 96 h and by CR47 treatment at 48 h after the treatment.

Key words: PR genes and proteins, PR induction, Clonostachys rosea, Fusarium culmorum, wheat.

Introduction

Pathogenesis-related proteins (PRs) are involved in plant systemic resistance against pathogen attacks (Van Loon & Van Strien, 1999), moreover they may be stimulated by antagonists such as Trichodema harzianum (De Meyer et al., 1998; Yedidia et al., 2000). Clonostachys rosea is known to be a mycoparasite of Fusarium culmorum (Pisi et al., 2001, Roberti et al., 2002) and is able to induce some kind of resistance in wheat seedlings (Roberti et al., 2001) The possible involvement of PR1, PR3 (chitinase), PR4 (wheatwins), PR5 (osmotin) and PR9 (peroxidase) proteins and genes in wheat systemic resistance induction by C. rosea 47 (CR47) has been investigated.

Material and methods

Wheat seeds cv. Creso were coated with C. rosea 47 and 4 days old coleoptiles were artificially inoculated with a plug of 10 days old F. culmorum (FC) colony. Seedlings were evaluated for disease symptoms 7, 8, 9 and 10 days after the treatment, corresponding to 24, 48, 72 and 96 h after inoculation. At each time, seedlings and roots were harvested and either RNA or total proteins were extracted. Peroxidase and chitinase activities were determined after isoelectrofocusing (IEF) in the 5-8 pH range, and after SDS-PAGE in the presence of glycol chitin, respectively (Trudel & Asselin, 1989; Caruso et al., 1999). The total chitinase activity was also detected by colorimetric determination of p-nitrophenyl derived from p-nitrophenyl-β-D-NN'-diacetylchitobiose cleavage (Roberts & Selitrennikoff, 1988). PR4 proteins were detected after immunoblot analysis carried out using anti-wheatwin1 polyclonal antisera,
whereas expression levels of PR1, PR4 and PR5 genes were evaluated by RT-PCR using specific primers.

**Results and discussion**

The presence of peroxidase isoforms in wheat coleoptiles was evaluated after isoelectric focusing. Activity staining showed the presence of several peroxidase isoenzymes ranging from pI 3.5 and 9.3 in all sample tested (Figure 1). Peroxidase activity increased during the time and it was more evident in roots (data not shown) than in coleoptiles. No appreciable difference was noted among root samples, whereas in coleoptiles some differences among samples treated with CR47 and FC were found. For instance, a peroxidase isoform (pI close to 6.3) was induced by FC inoculation (I) and enhanced in CR47-treated seeds inoculated with FC (T+I) 10 days from the treatment. This isoform was always reduced by CR47 treatment alone. This result is evident in the same Figure 1 on the right, where the densitometric analysis of peroxidase isoform with pI 6.3 is shown.

Figure 1. Peroxidase activity in wheat coleoptiles upon seed treatment with CR47 (T) and infection with FC (I) at 7 through 10 days from the treatment; C, untreated seeds - uninfected coleoptiles; T+I, treated seeds and infected coleoptiles; F, peroxidase profile of FC mycelium (left). Densitometric analysis of peroxidase isoform pI 6.3 (right)

Concerning chitinase activity, no significant difference was found among samples after SDS-PAGE separation, but some induction by FC (I) at 10 days and by CR47 treatment (T) at 8 days after the treatment, was revealed by colorimetric determination (Figure 2).

Figure 2. Time course of chitinase activity in wheat coleoptils determined by the release of p-nitrophenyl derived from nitrophenyl-β-D-NN’-diacetyl-chitobiose cleavage. C, I, T and T+I see Figure 1. Bars represent standard deviations.
Figure 3. Time course expression of wheat PR genes in response to CR47 treatment (T), *F. culmorum* infection (I) and *F. culmorum* infection on CR47-treated seeds (T+I). Coleoptiles and roots were harvested 7 (1), 8 (2) and 10 (3) days after treatment and infection as well as from control seeds (C). RT-PCR analysis was performed using specific primers. As control, actin mRNA was also amplified.

Figure 4. Time course expression of wheat PR4 protein in response to CR47 treatment (T), *F. culmorum* infection (I) and *F. culmorum* infection on CR47-treated seeds (T+I). Coleoptiles and roots were harvested 7 (1), 8 (2) and 10 (3) days after treatment and infection as well as from control seeds (C). Western blot analysis was carried out using anti-wheatwin1 antisera. Molecular markers (M) and wheatwin1 (W1) are indicated.
Transcripts corresponding to wheat PR4 genes were expressed at very low levels in both coleoptiles and roots harvested from control seeds (C) remaining unchanged throughout the period of sampling (Figure 3). On the contrary, transcript expression was induced in the same tissues upon FC infection either in control (I) or CR47-treated seeds (T+I). To determine whether other PR genes are co-ordinately activated with those belonging to the PR4 family, we extended the analysis to PR1 and PR5 genes. The time course expression pattern evaluated after RT-PCR analysis revealed that either PR1 or PR5 genes are constitutively expressed in both tissues independently from pathogen infection or CR47 treatment. Moreover, in order to investigate the expression pattern of PR4 proteins in the same tissues treated with CR47 alone or in combination with F. culmorum, western blot analyses were performed utilising the anti-wheatwin1 antisera (Figure 4). A rapid accumulation of PR4 proteins in all samples was evidenced, displaying an induction pattern similar to that observed at the transcriptional level. In conclusion, an induction of resistance in wheat coleoptiles with the antagonist along with the pathogen can be hypothized, as spatial and temporal CR47 seed application and FC inoculation were different.

Acknowledgements

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References


Seed treatments for organic vegetable production

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Due to the difficulty in organic farming of producing pathogen free seeds, and the lack of simple, effective non-chemical methods for seed sanitation, a substantial part of the seed used by European organic vegetable growers is still derived from conventional production. This will be strongly restricted after the year 2003 (EU Council regulation 2092/91). In March 2003, a EU-project "Seed Treatments for Organic Vegetable Production" (QLRT-2002-02239; STOVE) was initiated. The project is aimed at improving currently available, non-chemical methods for control of seed-borne vegetable pathogens and to develop new methods, which are acceptable to organic farming. The participants are Federal Biological Research Centre for Agriculture and Forestry (BBA), Germany (M. Jahn, E. Koch, C. Kromphardt, A. Schmitt); Plant Research International (PRI), Wageningen, Netherlands (S. Groot, J. v.d. Wolf); Swedish University of Agricultural Sciences (SLU), Uppsala, Schweden (G. Forsberg, B. Gerhardson); University of Gothenburg, Sweden (T. Amein, S. Wright); Findus R&D AB, Bjuv, Sweden (R. Stegmark, M. Wikström); Nunhems Zaden BV, Haelen, Netherlands (J. v.d. Berg); Nunhems Zaden (Hild), Marbach, Germany (M. Mistele, S. Werner); University of Turin, (Agrinnova) Italy (M. Gullino, F. Tinivella); Horticulture Research International (HRI), Wellesbourne, Great Britain (S. Roberts). Together with three physical methods (hot water, hot air and electron treatment), microorganisms and other agents of natural origin acceptable to organic farming will be included in the project. Initially, the three physical methods are being adapted for different vegetable species (e.g. carrot, parsley, cabbage, lamb’s lettuce, basil, bean) and their respective seed-borne pathogens (e.g. *A. dauci*, *S. petroselini*, *X. campestris*, *P. valerianellae*, *Fusarium* spp., *C. lindemuthianum*). In parallel, potential alternative seed treatments (micro-organisms, plant extracts, inducers of resistance) are also being tested. The efficacy of the methods will be compared in glasshouse and field trials, and selected combinations will be evaluated. Special regard will be placed on physiological factors determining the sensitivity of seeds towards the physical methods. The results of the first experiments were presented. (See http://www.stove-project.net/ for further information).
Antibacterial activity of some essential oils

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Abstract: Essential oils extracted from fruits of coriander, cumin, caraway and wild fennel were assayed in vitro for antibacterial activity toward two laboratory and 29 phytopathogenic bacterial species and bacteria that cause diseases of cultivated mushrooms. Significant antibacterial activity, as determined with the agar diffusion method, was shown against Gram positive and Gram negative bacteria. A reduced effect was observed for the wild fennel oil. The above essential oils may be potential bactericides useful for the control of plant and mushrooms bacterial diseases.

Keywords: Bactericides, plant pathogenic bacteria control; bacterial diseases of cultivated mushrooms, Coriandrum sativum L., Cuminum cyminum L., Carum carvi L., Foeniculum vulgare Miller var. vulgare (Miller).

Introduction

The control of bacterial diseases of plants, which may be destructive under certain environmental conditions, is a considerable challenge in the agriculture practice because of the limited availability of bactericides (Claflin, 2003). Besides the antibiotics and copper compounds which for some extent present undesirable attributes either for human and animal health or for the environment, no other active principles for the bacterial plant disease control are available. In particular, antibiotics are actually forbidden in the agricultural practices in many countries to avoid the possible selection of individual bacteria resistant to the drugs and hence the possible horizontal genetic transfer to animal and human pathogens (McManus et al., 2002). In addition, also the use of copper compounds, because of their general toxicity and mainly for the impact on the environment, is on the way to be restricted and controlled in the European Union through rule n° 473/2002.

The request of reduction in the use of the pesticides in agriculture prompts the need for the development of alternative active compounds and/or methods for the control of plant bacterial diseases to be used in integrated crop management as well as in bio-organic agriculture. The search for natural substances possibly harmless for the consumers and for the environment and useful for the protection of crops from diseases has become a foreground need. Many studies have pointed out on the possibility to use the essential oils and/or their components in medical and plant pathology as well as in the food industry for the control of pathogens and micro-organisms responsible for food spoilage. The objective of this study was to evaluate the antibacterial activity in vitro of essential oils toward bacteria responsible of diseases on plants and cultivated mushrooms.
Materials and methods

Bacterial cultures
One to four strains of each bacterial entity reported in Table 1 were used. Subcultures were obtained by growing bacteria for 48 - 72 h on the medium B of King (KB; King et al., 1954) in the case of pseudomonads and on WA (sucrose 10 g/l, bacto-peptone 5 g/l, K2HPO4 0.5 g/l, MgSO4x7H2O 0.25 g/l, agar 18 g/l) (Koike, 1965) for the other bacterial species.

The source of bacterial strains was: NCPPB, National Collection Plant Pathogenic Bacteria (UK); ICMP, International Collection of Microorganisms from Plants (Auckland, New Zealand); GSPB, Gottinger Sammlung Phイトopathogene Bakterien (Gottingen, Germany); XCPFu4487, bacterial strain supplied by Dr. L.E. Claflin, (Department of Plant Pathology, Kansas State University, USA); ITM, Istituto Tossine e Micotossine (Bari, Italy); DPP, Dipartimento di Protezione delle piante (Università della Tuscia, Viterbo, Italy); IPV-BO, Istituto di Patologia Vegetale (Università di Bologna, Italy); DAPP-PG, Dipartimento di Arboricoltura e Protezione delle Piane (Università degli Studi di Perugia, Italy); USB, Università degli Studi della Basilicata (Potenza, Italy).

Isolation and analysis of essential oils
Aliquots of 25 g of dried fruits of coriander (Coriandrum sativum L.), wild fennel [Foeniculum vulgare Miller var. vulgare (Miller)], cumin (Cuminum cyminum L.) and caraway (Carum carvi L.) were grounded and then the resulting powder was submitted to hydrodistillation for 3 h following the procedure reported in the European Pharmacopoeia (Anonymous, 1997). The essential oils were analysed by GC and GC-MS as previously described (Senatore & Rigano, 2001). The oil components were identified by calculating the Kovats index and by comparing mass spectra with those reported in literature (Adams, 1995) and in the GC/MS data base (NIST 98 e Wiley-5). Co-injection was made with standard components.

Disc diffusion assay
Ten µl 1:1 serial dilutions in methanol of stock solutions of each essential oil, obtained in 80% (v/v) methanol, and of 1.6 mg/ml of rifampicin were added to 6 mm diameter sterile blank disks previously deposited onto the surface of Petri plates containing 10 ml of KB or WA (0.7% agar) depending on the bacterial species. Aliquots of target bacteria suspensions were added to the above media, maintained at 45°C, to obtain a final population of about 107 cfu/ml. After 48 hours incubation at 25°C the minimal inhibitory quantity (MIQ), expressed in µl for essential oils or µg for rifampicin, which causes an apparent inhibition zone around the 6 mm diameter disks was recorded. The assays were performed twice with three replicates.

Results and discussion
The Gas-Chromathography analysis showed the complex composition of essential oils of caraway, coriander, cumin and wild fennel. Beside a high number of minor or trace substances, the main components of essential oils were linalool (64.5%) for coriander oil, p-mentha-1,4-dien-7-al (27.4%) and cumin aldehyde (16.1%) for cumin oil, carvone (23.3%) and limonene (18.2%) for caraway oil and anethole (59.2%) for wild fennel oil.

The antibacterial activities of essential oils assayed in vitro for antibacterial activity toward two laboratory and 29 phytopathogenic bacterial species as well as responsible of cultivated mushrooms diseases, are reported in Table 1. A significant antibacterial activity was shown against Gram positive and Gram negative bacteria belonging to Clavibacter,
Curtobacterium and Rhodococcus, and Erwinia, Xanthomonas, Ralstonia and Agrobacterium genera, respectively. A much reduced effect was observed for the wild fennel oil.

Table 1. Minimal inhibitory quantity (MIQ) of coriander (A), cumin (B), caraway (C) wild fennel (D) essential oils against bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>N° of strains</th>
<th>MIQ (µl)a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram negative</strong></td>
<td></td>
<td>A  B  C  D</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>1</td>
<td>1  4  1  Na</td>
</tr>
<tr>
<td><em>Pseudomonas syringae pv. phaseolicola</em></td>
<td>4</td>
<td>3  3  2  Na</td>
</tr>
<tr>
<td><em>P. s. pv. pisi</em></td>
<td>2</td>
<td>3  6  6  Na</td>
</tr>
<tr>
<td><em>P. s. pv. syringae</em></td>
<td>3</td>
<td>4  2  2  Na</td>
</tr>
<tr>
<td><em>P. s. pv. aptata</em></td>
<td>2</td>
<td>4  8  3  Na</td>
</tr>
<tr>
<td><em>P. s. pv. api</em></td>
<td>1</td>
<td>ND  2  2  Na</td>
</tr>
<tr>
<td><em>P. s. pv. atrofaciens</em></td>
<td>2</td>
<td>8  1  1  4</td>
</tr>
<tr>
<td><em>P. s. pv. lachrymans</em></td>
<td>2</td>
<td>Na  1  1  Na</td>
</tr>
<tr>
<td><em>P. s. pv. maculicola</em></td>
<td>2</td>
<td>1  3  3  Na</td>
</tr>
<tr>
<td><em>P. s. pv. tomatophthora</em></td>
<td>2</td>
<td>4  8  6  Na</td>
</tr>
<tr>
<td><em>P. s. pv. glycinea</em></td>
<td>2</td>
<td>1  1  1  1</td>
</tr>
<tr>
<td><em>Pichia cichorii</em></td>
<td>1</td>
<td>8  8  8  Na</td>
</tr>
<tr>
<td><em>P. viridiflava</em></td>
<td>2</td>
<td>Na  Na  Na  Na</td>
</tr>
<tr>
<td><em>P. corrugata</em></td>
<td>1</td>
<td>4  8  1  Na</td>
</tr>
<tr>
<td><em>P. tolaasii</em></td>
<td>1</td>
<td>Na  1  1  8</td>
</tr>
<tr>
<td><em>P. reactans</em></td>
<td>1</td>
<td>Na  4  4  Na</td>
</tr>
<tr>
<td><em>P. agarici</em></td>
<td>1</td>
<td>4  4  1  Na</td>
</tr>
<tr>
<td><em>Erwinia carotovora subsp. carotovora</em></td>
<td>1</td>
<td>0.5  2  1  8</td>
</tr>
<tr>
<td><em>E. c. subsp. atroseptica</em></td>
<td>1</td>
<td>0.5  0.5  0.5  8</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>2</td>
<td>0.5  0.75  0.75  3</td>
</tr>
<tr>
<td><em>Burkholderia gladioli pv. agaricicola</em></td>
<td>1</td>
<td>4  8  4  8</td>
</tr>
<tr>
<td><em>Xanthomonas campestris pv. phaseoli</em></td>
<td>3</td>
<td>0.25  0.62  0.18  0.5</td>
</tr>
<tr>
<td><em>X. c. pv. phaseoli var. fuscans</em></td>
<td>4</td>
<td>0.25  0.5  0.5  0.75</td>
</tr>
<tr>
<td><em>X. c. pv. vesicatoria</em></td>
<td>4</td>
<td>0.25  0.37  0.25  1.5</td>
</tr>
<tr>
<td><em>X. c. pv. campestris</em></td>
<td>1</td>
<td>0.25  1  0.5  6</td>
</tr>
<tr>
<td><strong>Gram positive</strong></td>
<td></td>
<td>A  B  C  D</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>1</td>
<td>0.5  1  0.5  Na</td>
</tr>
<tr>
<td><em>Clavibacter michiganensis subsp. michiganensis</em></td>
<td>2</td>
<td>0.43 0.14 0.28 8</td>
</tr>
<tr>
<td><em>C. m. subsp. sepedonicus</em></td>
<td>1</td>
<td>0.5  0.5  0.5  1</td>
</tr>
<tr>
<td><em>Curtobacterium flaccunfaciens pv. flaccunfaciens</em></td>
<td>2</td>
<td>0.5  0.5  0.5  Na</td>
</tr>
<tr>
<td><em>C. f. pv. betae</em></td>
<td>2</td>
<td>0.75 0.5  0.75  Na</td>
</tr>
<tr>
<td><em>Rhodococcus fascians</em></td>
<td>2</td>
<td>0.5  0.5  1  2</td>
</tr>
</tbody>
</table>

a MIQ, average volume needed for the bacterial growth inhibition; Na, the deposition of 8 µl of essential oils on sterile blank disks did not lead to an inhibition zone; ND, not determined.

Data on the quantitative composition of the essential oils would suggest that the antibacterial activity may be due to the main components already known for their antimicrobial activity. For example, the highly antibacterial activity of coriander essential oil may be probably due to the high concentration of linalool (64.5%), a compound well-known for the bactericide properties (Scortichini & Rossi, 1991; Kim et al., 1995). However, also α-pinene (5.1%), nerol (4.6%) and limonene (3.6%), known for their bactericide properties (Scortichini & Rossi, 1991; Dorman & Deans, 2000), may be important. Antibacterial activity of the cumin essential oil is probably due to the high content of cumin aldehyde (16.1%) and
β-pinene (11.4%) both known for their antimicrobial properties (Scortichini & Rossi, 1991; Dorman & Deans, 2000). Antibacterial activity of the caraway essential oil appear to be due to the presence of the high levels of carvone (23.20 %) and limonene (18.2 %) already reported for the capability to inhibit the growth of fungi and bacteria (Scortichini & Rossi, 1991 Dorman & Deans, 2000).

The limited antibacterial activity shown by the wild fennel essential oil may be probably due to anethole (59.2%), characterised by a moderate antimicrobial activity (Kubo & Himejima, 1991; Himejima & Kubo, 1993), and to the significant presence of limonene (15.0%) and α-pinene (2.7%) known for their ability to inhibit the bacterial growth (Scortichini & Rossi, 1991; Dorman & Deans, 2000). However, also the low or trace components (i.e. sabinene, and eugenol, borneol, α-terpineol, gerianol, thymol and geranil acetate, known for their bactericide properties (Scortichini & Rossi, 1991; Kim et al., 1995; Dorman & Deans, 2000), may contribute, either synergistically or in an antagonistic manner, on the antibacterial activity of the essential oils.

The significant activity of essential oils against bacterial pathogens of important crops suggest the possibility to use the above substances for their control and, in particular, in a biorganic agriculture. The availability of new active principles such as essential oils or their components in the disease control practice is of great interest. Of particular interest is the possibility to use the above substances for seeds treatment. Of course, other studies are necessary to evaluate the toxicity of the above substances toward seeds and/or plants and to set the appropriate formulations useful for the purpose.

References

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Management of soil borne diseases
Evaluation of *Trichoderma* strains as biocontrol tools for integrated management of strawberry root rot

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**Abstract:** *Trichoderma harzianum* strain T22 and *T. hamatum* strain T382 were tested as biocontrol amendments for strawberry production. Runner tips were planted in potting mix amended with T22, T382, or neither, and maintained in a greenhouse for four weeks. The resulting plug transplants were evaluated for root health (root rot severity, abundance of feeder roots) and plant growth (leaf area, dry weight of leaves and roots). Transplants were established in field soil treated with fumigant (Telone C35), compost, compost with T382, or untreated. Field plants were sampled throughout the season and assessed for root health, plant growth, and yield. Fungi were isolated from roots of transplants and field plants. *Trichoderma* populations were monitored in field soil. In the greenhouse, transplant treatments did not affect root health but T382 improved leaf area and root dry. Root rot pathogens were isolated less frequently from transplants amended with T22 or T382 than from untreated controls. T382 became established and maintained a stable population in field soil throughout the season. Transplant treatments did not significantly affect root health or plant growth in the field. Soil fumigation significantly reduced root rot, and increased plant growth and yield compared to the other treatments. Compost and compost with T382 offered no benefit compared to the untreated control.

**Keywords:** biological control, *Trichoderma*, compost, strawberry root rot, *Phytophthora*, *Pythium*, *Rhizoctonia*

**Introduction**

Strawberry root rot diseases are caused by a complex of pathogens, including *Rhizoctonia*, *Pythium* and *Phytophthora*, and can seriously reduce plant vigor and productivity (Wing et al., 1994). Root pathogens may be disseminated on infested transplants or may be present in infested fields. The goal of our research program is to develop and implement an IPM system for managing strawberry root rots with emphasis on biological approaches. *Trichoderma* species are well known for their biocontrol activity against soil borne pathogens (Elad et al., 1981; Harman, 2000; Chung & Hoitink, 1990). The objective of this study was to evaluate the effectiveness of two *Trichoderma* biocontrol strains in suppressing root rot and promoting plant growth and productivity on strawberry.

**Materials and methods**

**Transplant production**

Strawberry runner tips (cv. Chandler) were planted on September 2002 in potting mix amended with the biocontrol agents *T. harzianum* strain T22 (Rootshield®), *T. hamatum* strain T382 (Chung and Hoitink, 1990), or neither (control). T22 was added to potting mix at a rate of 6x10⁴ cfu /g dry weight. Potting mix was amended with T382 by adding 5% (v/v)
composted cow manure containing $2 \times 10^5$ cfu T832/g dry weight. The tips were grown under greenhouse conditions for four weeks to produce plug transplants. A subsample of transplants was evaluated for root rot severity, abundance of feeder roots, leaf area, and dry weight of leaves and roots. Fungi were isolated from roots of runner tips and plug transplants. The roots were rinsed in water, and healthy and diseased root pieces were plated onto selective media. Fungi were identified to genus based on morphological characteristics.

**Field production**

A field trial was established in Plymouth, North Carolina, on October 4, 2002 with the transplants not used for preplant assessments. The experimental design was a split-plot with four replications; field soil treatments were main plots and transplant treatments were split plots. Field soil was treated with fumigant (Telone C35, 1.4 m$^3$/ha), compost (cotton gin trash; 56.6 m$^3$/ha), compost with T382, or left untreated (control). For the compost with T382 treatment, compost was inoculated with T382 at a rate of $2 \times 10^5$ cfu/g dry weight, then incubated for two weeks. All treatments were applied to the soil one month before planting. Plants were destructively sampled five times during the field season (Dec 2002-May 2003) and evaluated for root health (rot severity; abundance of feeder roots) and plant growth (leaf area; dry weight of roots, crowns, leaves, flowers). Fungi were isolated from diseased and healthy root pieces as described above. The population of *T. hamatum* in field soil was quantified by dilution plating onto *Trichoderma* selective medium (Chung & Hoitink, 1990).

**Results**

**Transplant production**

Root health of transplants was not affected by treatments with T22 or T382 (Figure 1A) but T382 stimulated leaf area and root dry (Fig 1B and C). Both biocontrol agents inhibited pathogens naturally occurring on roots. The percentage of fungal isolation from roots of runner tips prior to treatment application was: *Rhizoctonia* (0%), *Fusarium* (50%), *Pythium* (10%), *Phytophthora* (7.5%) and *Trichoderma* (0%). Soil treatment with T382 suppressed *Phytophthora*, and both T382 and T22 reduced *Pythium* on transplant roots (Table 1). *Trichoderma* was more frequently isolated from roots of transplants treated with T22 than from the other treatments (Table 1).

![Figure 1](image-url)  
Figure 1. Effects of *Trichoderma* biocontrols on root health (A) and growth (B,C) of plug transplants grown in the greenhouse for four weeks. Root rot rating: 1 = no disease, 10 = 100% roots with rot. Feeder root rating: 1=none, 10=very abundant.
Table 1. Influence of *Trichoderma* biocontrols on fungal isolation from healthy and diseased roots of plug transplants grown in the greenhouse for four weeks. Values shown are percentage of roots from which each fungus was isolated.

<table>
<thead>
<tr>
<th>Fungal genus/Species</th>
<th>Diseased Roots</th>
<th>Healthy Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=19)</td>
<td>T22 (n=10)</td>
</tr>
<tr>
<td><em>Trichoderma</em></td>
<td>36.8</td>
<td>70.0</td>
</tr>
<tr>
<td><em>Rhizoctonia</em></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Fusarium</em></td>
<td>5.3</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Phoma</em></td>
<td>5.3</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Pythium (all spp)</em></td>
<td>57.9</td>
<td>20.0</td>
</tr>
<tr>
<td><em>P. irregulare</em></td>
<td>21.6</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Phytophthora cactorum</em></td>
<td>36.8</td>
<td>30.0</td>
</tr>
</tbody>
</table>

**Field production**

Transplant treatments showed no significant \( P \leq 0.05 \) effects in the field but plant growth, root health and yield were affected \( P \leq 0.05 \) by field soil treatments (Figure 2). Soil fumigation reduced \( P \leq 0.05 \) root rot (Figure 2A), increased leaf area (Figure 2B), and increased dry weights of leaves, crowns and roots (data not shown) compared to the other treatments. Soil amendments with compost and compost with T382 offered no benefit compared to the control. Marketable yield was greater \( P \leq 0.05 \) for fumigated plots that for the other treatments (data not shown).

*Rhizoctonia* and *Pythium* were isolated less frequently from roots of plants from soil treated with fumigant or compost with T382 than control plants (data not shown). The population of *T. hamatum* was up to 80-fold greater in field soil treated with compost inoculated with T382 than in other treatments (Figure 3).

![Figure 2](image-url)  
**Figure 2.** Mean root rot severity (A) and leaf area (B) of field plants for each soil treatment

**Discussion**

*Rhizoctonia*, *Pythium* and *Phytophthora* were isolated from roots of strawberry runner tips and plug transplants. These results corroborate the importance of pathogen dissemination on strawberry propagation material and support the need for production systems that generate...
clean transplants. Both *T. hamatum* T382 and *T. harzianum* T22 suppressed or reduced root pathogens on transplants. In addition, T382 stimulated root and leaf development. Similar disease suppressive and growth promoting effects by *Trichoderma* biocontrols have been previously reported on strawberry (Elad et al., 1981). *Trichoderma* applications to transplants may therefore be an effective tool to reduce the risk of pathogen dissemination into fields and improve transplant vigor.

*T. hamatum* became established and persisted in field soil when incorporated on inoculated compost. This suggests that stable populations of *Trichoderma* biocontrols can be maintained in field conditions, without the need for subsequent treatment applications, if the soil environment is adequately managed. In our study, compost may have served as substrate for colonization and survival of the biocontrol agent (Hoitink & Boehm, 1999).

Soil fumigation reduced root rot, and increased plant growth and yield but the presence of *T. hamatum* T382 in field soil showed no beneficial effect on the plants. Applications of other *Trichoderma* strains to strawberry fields suppressed root rots and increased yield (Elad et al., 1981). It is not clear if our results were directly related to poor effectiveness of the T382 since this biocontrol was added with compost to field soil. In previous studies (Grabowski, 2001), compost amendments resulted in yields equal or higher than soil fumigated with methyl bromide but not in this trial where are alternate source of compost was used. This suggests that compost composition is highly variable and can confound results (Hoitink & Boehm, 1999).

Finally, although *Trichoderma* applications were beneficial for transplant production, those treatment effects did not persist in the field in our study, even when transplants were planted in fumigated soil. We conclude that management of strawberry root rot should rely primarily on effective field treatments.

**Figure 3. Mean population of *T. hamatum* in field soil**

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Characterization of avocado root-colonizing bacteria antagonistic against *Rosellinia necatrix*

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Abstract: Forty-one different bacterial strains were isolated from the roots of symptomless avocado trees located in orchards of the southern coast of Spain affected by *Rosellinia necatrix*. The *in vitro* antagonistic capacity of these strains against *R. necatrix* was studied; ten of them could prevent fungal growth and none of them resulted to be a plant pathogen. We also analyzed two characteristics known to be involved in root colonization: a) motility; all of them showed at least two kinds of motility and, b) the production of N-acyl homoserine lactone (AHL) molecules involved in regulation by quorum-sensing (QS); three of the isolated strains showed this communication system. We have also determined the type of antagonistic compounds produced by the selected strains: lipases, β-glucanases, cellulases, proteases and volatile compounds (HCN); all the rhizobacterial strains, except one, showed at least two different enzymatic activities.

Key words: Root colonization, fungal diseases, Avocado Root Rot, *Rosellinia, Pseudomonas*

Introduction

One of the two most important diseases affecting avocado orchards in the south of Spain is the white root rot caused by *Rosellinia necatrix*, a disease restricted to Andalusian orchards. Characteristic symptoms of this disease are rotting of roots, yellowing and falling of leaves, wilting and, finally, death of the tree. Dispersal of *R. necatrix* occurs mainly by mycelial strands and by contact between diseased and healthy roots.

Biological control of fungal diseases is usually based on the application of microorganisms selected by their capacity to produce *in vitro* antifungal factors, however, recent studies about bacterial behavior in natural environments showed the importance of the competence with other microorganisms for an efficient colonization of a given ecological niche. In this sense, efficient colonization of the root system by the applied bacteria is required for biological control of soil-borne diseases. This project is directed to the isolation of bacterial strains showing antagonism versus *R. necatrix* and colonizing efficiently the root system of avocado plants.

Material and methods

*Microorganisms and growth conditions*

*R. necatrix* strain CH 53 was isolated from the roots of naturally diseased avocado orchards (Pérez-Jiménez et al, 2003). *Escherichia coli* containing the *lux*R gene and a fusion of the *lux*I promoter to a *gfp* allele (gfp-ASV) encoding an unstable version of this protein was used for detection of AHLs molecules as described by Andersen et al. (2001).
Root-colonizing bacterial strains were isolated as follows. The root system of avocado plantlets obtained from in vitro germinated Topa-Topa seedlings was inoculated with a bacterial suspension extracted with phosphate buffer from roots of symptomless avocado trees located in Andalusian orchards affected or not by \textit{R. necatrix}. After ten days of growth, roots tips were blended in phosphate buffer and different dilutions were plated onto KB medium. After several days of incubation at 25ºC, bacterial colonies showing different morphologies were isolated and classified according to the pattern of fluorescence emission, the induction of HR in tobacco plants, and the API 20NE test (BioMerieux). \textit{R. necatrix} and the bacterial strains isolated from avocado roots were grown on KB or LB media at 24ºC; \textit{E. coli} was grown on LB medium at 37ºC.

\textit{In vitro} antagonistic activity against \textit{R. necatrix}
Antagonistic activity of the isolated bacterial strains against \textit{R. necatrix} was performed by the method previously described by Woeng et al. (1998)

Characterization of antifungal metabolites
Bacterial strains were tested as previously described for production of HCN (Castric, 1975), proteases (Woeng et al., 1998), lipases (Howe, 1976), cellulases (Hankin, 1977), and \( \beta \)-glucanases (Walsh et al., 1995).

Motility assays
Swimming and swarming motility assays were carried out as described by Sánchez-Contreras et al. (2001). Twitching motility was performed on KB medium plates containing 1% of agar; the bacterial cells were stabbed through the agar with a wooden picker to the bottom of the Petri dish. After three days of incubation at 25ºC, the agar was removed gently from the plate and the cells were stained for 1 minute using crystal violet.

Results and discussion

\textit{Isolation and characterization of root-colonizing avocado and antagonistic bacteria}
Forty-one different bacterial strains were isolated from the root system of \textit{in vitro} avocado plantlets as described above. Ten of them (24.4 \%) inhibited \textit{in vitro} growth of \textit{R. necatrix} and were classified according to the pattern of fluorescence emission and the API test. Strain GBF.2.2 clearly emitted fluorescence and was identified as \textit{Pseudomonas fluorescens} by the API test. Strain GBF.2.1 was identified as \textit{P. malthophilia}; a bacterial strain belonging to this species and showing chitinolitic activity, has been used for biological control of \textit{Bipolaris sorokiniana} in turfgrass. The rest of the strains could not be clearly assigned by these tests to any bacterial genus; currently, we are trying to identify them by 16S rDNA sequencing. None of the ten selected strains were classified by the API test as plant pathogens or showed hypersensitive response in tobacco plants.

\textit{Production of hydrolytic enzymes and volatile compounds}
With the exception of strain GBF.2.17 that showed only \( \beta \)-glucanase activity, all the other strains showed at least two different activities, being more frequent the production of cellulases and \( \beta \)-glucanases (7 strains). Strains GBF.1.1 and GBF.1.11 showed four different activities, being good candidates for further studies. Proteases, \( \beta \)-glucanases and cellulases are cell-wall degrading enzymes known to play a role in the mechanism of action of antagonistic microorganisms. Two of the isolated bacteria, GBF.2.2 and GBF.2.10, produced HCN which is a broad-spectrum antimicrobial compound involved in biological control of root diseases by many plant-associated fluorescent Pseudomonads. Currently, we are testing the production of other metabolites known to be involved in biological control such as the production of siderophores and antibiotics.
Table 1. Production of antifungal metabolites by strains inhibiting in vitro growth of *R. necatrix*

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Lipases</th>
<th>Proteases</th>
<th>β-glucanases</th>
<th>Cellulases</th>
<th>HCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBF1.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>GBF1.3</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>GBF1.5</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>GBF1.11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>GBF1.18</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>GBF2.2</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>GBF2.10</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>GBF2.13</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+/-</td>
<td>–</td>
</tr>
<tr>
<td>GBF2.17</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GBF2.18</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

–: Absence; +: Presence; +/-: Weak Activity

**Surface motility of antagonistic colonizing strains**

Surface motility confers potential benefits to bacteria in the rhizosphere including increased efficiency in nutrient acquisition, avoidance of toxic substances, ability to translocate to preferred hosts and access to optimal colonization sites within them (Andersen, 2003). In this sense, surface motility of root-associated biocontrol bacteria might play an important role in the effective protection of plant-roots against fungal attack. In a screening for the motility of the selected strains, three types of motilities were assayed (Figure 1). We found that GBF.1.3, GBF.1.5, GBF.1.18, GBF.2.13, GBF.2.17 revealed the three types of motilities, the rest showed two of them; e.g. GBF.1.1 and GBF.1.11 showed swimming and twitching motilities while GBF.2.2 and GBF.2.10 showed swarming and twitching motilities.

![Figure 1. Surface motility phenotypes. A, GBF.1.11 swimming motility presented concentric haloes; B, GBF.2.2 swarming motility presented a dendritic pattern; C, GBF.1.11 twitching motility.](image)

**Production of N-Acyl homoserine-lactones**

Quorum-sensing regulation, mediated by AHLs molecules among other compounds, regulates a variety of physiological processes, including bioluminescence, swarming, swimming and twitching motility, production of antifungal compounds and root colonization. Three of the
isolated strains, GBF.1.11, GBF.2.10, GBF.2.17, produced AHLs that could be detected by the used monitoring strain (Figure 2).

Figure 2. A, Schematic representation of AHLs detection. B, An epifluorescence image of the AHL sensor JM105 harboring plasmid pJBA132 platted against the AHL-producing strain GBF.2.10.

Conclusions

A high percentage (24.4%) of the bacterial strains selected by their ability to colonize the root of avocado trees showed the capacity to inhibit *in-vitro* growth of the fungi *R. necatrix*. Among the antifungal activities tested, production of β-glucanases and cellulases were the most abundant (seven strains out ten), closely followed by the production of proteases (six strains). Strains GBF1.1. and GBF1.11 showed four out of the five antifungal activities tested and they will be tested for their ability to control *in-vivo* the growth of *R. necatrix* in avocado roots.

References


Integrated management of *Pythium* root rot in flower bulb production

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Root rot caused by fungi of the genus *Pythium* is an important disease in flower bulb cultivation in the Netherlands. Measures to control this disease such as fungicides do not always result in consistent disease control. In this study, several control methods are tested in single and combined applications, to investigate compatibility and synergism of these methods. Control methods, which are being studied at this moment, are antagonistic *Pseudomonas* bacteria, biofumigation, fungicides and cultural measures.

Antagonistic *Pseudomonas* spp. strains producing the antibiotic 2,4-diacetylphloroglucinol (Phl) or producing biosurfactants showed *Pythium* root rot control of different bulb crops (*Iris*, *Crocus*, *Hyacinthus*) in pot experiments under controlled conditions. A biosurfactant-producing strain almost completely prevented *Pythium* root rot in *Hyacinthus* and *Crocus*. A mutant strain, lacking the biosurfactant-production, did not suppress root rot in *Hyacinthus* indicating that this biosurfactant compound is involved in disease control. In bioassays under field conditions both the biosurfactant-producing strain and a Phl-producing strain were able to control disease of different crops (*Hyacinthus*, *Iris* and *Crocus*). In several expanded field trials both strains were able to control of *Pythium* root rot but these results vary per field and year to year. Furthermore we are investigating the possibility of using a biofumigation crop to reduce *Pythium* inoculum in the soil before planting the bulbs. So far we have obtained promising results under field conditions.

Since several measures tested in single applications do not result in sufficient *Pythium* root rot control we are developing an integrated strategy to manage this disease. At the moment integrated control of *Pythium* is investigated in field experiments with naturally infested soil. In these field experiments single and combined applications of *Pseudomonas* spp. strains, a biofumigation crop and standard and lower dosages of a pesticide are compared.
Role of the antifungal compounds produced by *Pseudomonas fluorescens* PCL1606 in the biocontrol activity of avocado white root rot

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Abstract: The bacterial strain *Pseudomonas fluorescens* PCL1606 showed biocontrol activity against *Rosellinia necatrix*, the causal agent of the white root rot of avocado trees. This bacterium displays a high antagonism against many phytopathogenic fungi by production of antifungal compounds, including the antibiotic 2-hexyl-5-propyl resorcinol (HPR). The role of HPR production in the biocontrol activity of *P. fluorescens* PCL1606 is being studied by constructions of Tn5 mutants impaired in this antagonistic trait and by analysis of the inserted and sequences of selected mutants.

Key words: *Rosellinia necatrix*, *Persea americana*, 2-hexyl-5-propyl resorcinol

Introduction

*Rosellinia necatrix* Prill. (anamorph: *Dematophora necatrix* Harting) is the causal agent of the white root rot disease in avocado (*Persea americana* Mill.) and many other crops (Sztejnberg & Madar, 1980). Control of the white root disease of avocado is complex. Solarization has showed protective results against *R. necatrix* in some cases (López-Herrera et al., 1998), and other approaches, as chemical fungicides or tolerant rootstocks are still experimental. In addition, the applications of a *Trichoderma* spp. showed good results in greenhouse experiments, but give it variable on field tests. Few other approaches have been tested to use other microorganisms as biocontrol agents.

For this, the use of bacterial strains could be an alternative biocontrol treatment, which can improve the integrated management of the white root rot. The strain *Pseudomonas fluorescens* PCL1606 has been selected in previous studies (Cazorla et al., 2001) because of its antagonistic activity against a very broad spectrum of phytopathogenic fungi, and because it displays biocontrol activity against *R. necatrix* in growth chamber experiments. Moreover, detailed studies about the antifungal compounds produced by *P. fluorescens* PCL1606 showed that it produces proteases, lipases and the antibiotic 2-hexyl-5-propyl resorcinol (HPR).

Materials and methods

Bacterial and fungal strains

*Pseudomonas fluorescens* PCL1606 and other *Pseudomonas* spp. strains were grown on KB media at 24°C for 48 h. *Rosellinia necatrix* Rn400 strain was isolated from an avocado tree
with white root located in southern Spain (Pérez-Jiménez, 1997). Strain ZUM2407 of Fusarium oxysporum f. sp. radicis-lycopersici was used for the tomato/Fusarium biocontrol test system.

Detection of antifungal metabolites
Detection of antifungal antibiotics in defective mutants was performed by TLC analysis of extracts of supernatants from bacterial cultures (Chin-A-Woeng et al., 1998; Sarguinet et al., 2000). Strains were also tested for production of HCN (Castric, 1975), and exoenzymatic activities including proteases (Chin-A-Woeng et al., 1998), lipases (Howe and Ward, 1976), β-glucanases (Walsh et al., 1995) and cellulases (Hankin & Anagnostakis, 1977).

Molecular analysis
Mutants impaired in antagonistic activity against phytopathogenic fungi were constructed by conjugation and transformation of Pseudomonas fluorescens PCL1606 with the plasmid pRL1063a, which harbours a Tn5 transposon with the reporter genes luxAB (Wolk et al., 1991). Flanking regions of the genes interrupted by the transposon in the different mutants were excised and sequenced. The obtained sequences were compared with those in the data base (Altschul et al., 1990).

Biocontrol tests
The bicontrol strains P. fluorescens WCS365 and P. chlororaphis PCL1391 were used as controls in the experiments (Simons et al., 1996; Chin-A-Woeng et al., 1998). Avocado/Rosellinia necatrix bioassays were performed using plants obtained by germination of embryos as described previously (Pliego-Alfaro et al., 1987). The bacterized plants were sown in vermiculite with wheat seeds infected with R. necatrix as inoculum (Cazorla et al., 2001; Freeman et al., 1986). Tomato/Fusarium oxysporum f. sp. radicis-lycopersici bioassays were performed using the experimental setup described previously (Chin-A-Woeng et al., 1998). Briefly, seeds were coated with bacteria and sown in pots containing potting soil infected with spores of F. oxysporum f.sp. radicis-lycopersici. In both cases, plants were grown in a greenhouse, and during 16 days the plants were examined for disease symptoms. Data were analyzed for significance using analysis of variance followed by Fisher’s least significant difference test (α=0.05), with SPSS software (SPSS Inc., Chicago, IL, USA). All experiments were performed at least twice.

Figure 1. Biocontrol ability of selected rhizobacterial isolates in the avocado/Rosellinia system. PCL1606: Pseudomonas fluorescens PCL1606; PCL1391: P. chlororaphis PCL1391; WCS365: P. fluorescens WCS365. Values with different letter
indications denote a statistically significant difference ($P \leq 0.05$).

Table 1. Homology of the flanking regions interrupted by the transposon in the different mutants impaired in the antagonistic activity. Study of some of them are in process.

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Homology to (%)</th>
<th>Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL1613</td>
<td>Hydrolases or acyltransferases (87%)</td>
<td><em>P. fluorescens</em> Pf0-1</td>
</tr>
<tr>
<td>PCL1614</td>
<td>Aminomethyltransferases (96%)</td>
<td><em>P. fluorescens</em> Pf0-1</td>
</tr>
<tr>
<td>PCL1615</td>
<td>ABC-type transporter</td>
<td><em>P. fluorescens</em> Pf0-1</td>
</tr>
<tr>
<td>PCL1616</td>
<td>In progress</td>
<td></td>
</tr>
<tr>
<td>UMAF8414</td>
<td>In progress</td>
<td></td>
</tr>
<tr>
<td>UMAF8418</td>
<td>In progress</td>
<td></td>
</tr>
<tr>
<td>UMAF8419</td>
<td>In progress</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Biocontrol activity of the wild type *P. fluorescens* PCL1606 and its derivative mutants impaired in antagonistic activity on (A) the avocado/*Rosellinia* and (B) tomato/*Fusarium* biocontrol test systems. Values with different letter indications denote a statistically significant difference ($P \leq 0.05$).

**Results and discussion**

*Pseudomonas fluorescens* PCL1606 displays biocontrol activity against *R. necatrix* (Figure 1). Analysis of the antifungal compounds produced by *P. fluorescens* PCL1606 showed the production of proteases and lipases activities, and the antibiotic 2-hexyl-5-propyl resorcinol (HPR). From 4700 constructed mutants, 7 were impaired in the antagonistic activity against phytopathogenic fungi. The selected mutants only produce proteases and lipases but do not produce HPR, and its absence was confirmed by TLC experiments. All selected mutants were similar to wild type *P. fluorescens* PCL1606 when they were characterized for their growth in minimal media and biochemical characteristics. Flanking regions of the interrupted gene by the transposons in each defective mutant were sequenced and compared with those in the data base (Table 1).

Then, biocontrol experiments showed that the mutant derivatives from the wild type strain *P. fluorescens* PCL1606 displays a reduction of the protection against *R. necatrix*, suggesting that production of HPR it is an important trait for the biocontrol activity of *P.*
fluorescens PCL1606. However, the reduction in biocontrol activity was not complete, indicating that more than one trait could take place in the biocontrol activity of *P. fluorescens* (Figure 2).

Acknowledgements

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Exploitation of spent mushroom compost in biological control against melon *Fusarium* wilt disease

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Abstract: The ability of mature spent mushroom compost (MSMC) fortified or not with Ca-Lignosulphonate (LS) and *Trichoderma atroviride* TA 312B2 to suppress the disease caused by *Fusarium oxysporum* f. sp. *melonis* was evaluated on melon plants under controlled conditions. The effect of LS on some chemical and microbial parameters of compost was also investigated. The addition of Ca-Lignosulphonate increased β-glucosidase activity and NH$_4^+$-N content. Melon plants grown in pot mixtures containing spent mushroom compost (30%) showed a significant reduction of disease severity compared to that of plants developed in the absence of the compost product. The suppressiveness of MSMC, MSMC + LS and MSMC + TA 312B2 was similar. The addition of Ca-Lignosulphonate along with antagonistic fungus to spent mushroom compost significantly enhanced its suppressive ability.

Key words: spent mushroom compost, Ca-Lignosulphonate, *Trichoderma atroviride*, β-glucosidase activity, microbial activity, Fusarium wilt disease, melon

Introduction

Spent mushroom (*Agaricus bisporus*) compost derived from wheat straw-bedded horse manure is a low cost organic product largely available in our country. This compost was suitable for the establishment of the antagonistic *Trichoderma atroviride* TA 312B2 strain (Montanari et al., 2004).

Calcium-Lignosulphonate is a by-product of the acid sulfite pulping process. This compound has a lignin-like structure: molecules are spherical in shape with negatively charged sulphonate, hydroxyl, phenolic and carboxyl groups (Soltani et al., 2002). It contains monosaccharides (20%) and polysaccharides (5%). Lignosulphonate significantly increased NH$_4^+$-N level in fertilizer mixtures in soil (Meier et al., 1993), and showed direct suppressive effect against several soil-borne plant pathogens (Lazarovitis, 2001). Mature spent mushroom compost with high ammonium content was highly favourable to *T. atroviride* TA 312B2 survival (Montanari et al, 2004). In previous *in vitro* tests carried out in our laboratory, a strong inhibition of *Fusarium oxysporum* f.sp. *melonis*, *Pythium* sp., *Sclerotinia* sp., and *Rhizoctonia solani* mycelial growth by Ca-Lignosulphonate was observed. On the contrary, mycelial growth, sporulation and conidial germination of *T. atroviride* TA312 B2 were not affected (data not shown).

The aim of this study was to investigate i) the effect of Ca-Lignosulphonate on some chemical and physical parameters, and some microbial and enzymatic activities correlated to suppressive ability of compost (Chen et al., 1988; Cotxarrera et al., 2002) ii) the suppressive effect of spent mushroom compost alone or fortified with Ca-lignosulphonate and TA 312B2 towards the disease caused by *Fusarium oxysporum* f.sp. *melonis* under controlled conditions.
Materials and methods

**Organic product and Ca-Lignosulphonate enrichment**
Mature spent mushroom (*Agaricus bisporus*) compost collected three months after steaming (MSMC) was used. The product was sieved through a 5 mm mesh, then liquid Calcium Lignosulphonate (LS, Bretax C®; Cartiere Burgo, Tolmezzo, Udine, Italy) containing 55% of solids was added (1%;v/w) to the half of compost (LS-MSMC). Sterile water was added to the other half of MSMC. Peat moss not enriched with LS (PM) was used as control.

All products were contained into black plastic bags separately, and maintained at 60% water content, 20-25°C, in the dark. Immediately before *Trichoderma* enrichment some physical and chemical properties of compost products were determined at the Laboratorio of Chimica del Suolo, University of Bologna.

**Trichoderma enrichment**
A conidial suspension of the benomyl-tolerant strain *T. atroviride* Karsten (TA 312B2) was introduced into each product to obtain a concentration of 8x10^5 conidia ml^-1 substrate. This fungus showed good antagonistic activity against *F. oxysporum* f.sp. *melonis* (FOM) in *in vitro* tests (data not shown). TA 312B2 population was evaluated 2 and 130 d after enrichment, immediately before the suppressiveness bioassay, using the serial dilution technique on selective media (Montanari et al., 2004).

**Microbial activity**
The microbial activity of MSMC and LS-MSMC with or without TA 312B2, and PM was monitored by measuring the rate of fluorescein diacetate hydrolysis (FDA) (Inbar et al., 1991). Fresh product samples (4 ml equivalent to 5 and 3 g dry wt of MSMC/LS-MSMC and PM, respectively) were placed in 250-ml Erlenmeyer flasks containing 20 ml sterile phosphate buffer, pH 7.6. The reaction was started by adding 0.2 ml of FDA. Each treatment consisted of 3 replicates and one blank. Flasks were shaken for 20 min on a rotary shaker at 25°C. The fluorescein concentration was determined using a Spectrophotometer and FDA was expressed as µg FDA hydrolyzed ml^-1 of organic product min^-1.

**Enzymatic assays**
Fresh product samples (0.6 ml equivalent to 300 and 100 µg dry wt of MSMC/LS-MSMC and PM respectively) were placed in 12 ml falcon tubes. 500 µl of 7mM *P*-nitrophenyl-β-acetylglucosamine (for chitinase determination), or 500 µl of 25mM *P*-nitrophenyl-β-D-glucopyranoside (for β-glucosidase determination), were added in tubes with 1 ml citrate-phosphate buffer (pH 5.8) and 1 ml triton-X 100 (0.2). Each treatment consisted of 3 replicates and one blank. Incubation was performed for 1 h at 30°C. *P*-nitrophenol (PNP) released by both enzymatic activities was measured using a Spectrophotometer. Enzymatic activity was expressed as µg PNP released ml^-1 of organic product h^-1.

**Suppressiveness bioassay**
A potting mixture constituted by MSMC, MSMC+TA 312B2, LS-MSMC and LS-MSMC +TA 312B2 mixed separately with sand and peat (1:1:1; v/v/v) was infected with a conidial suspension of FOM (10^5 conidia/ml) 140 d after the fortification of compost with LS and/or TA 312B2. A week later, 5 d old melon plants, were transplanted into each 400 ml plastic pot containing separately the different mixtures. Pots were maintained in a climate cell at 27°C, 12 h day light period and 70-80% RH for 4 weeks. Melon plants growing into peat-sand mixture (PS; 2:1; v/v) inoculated with FOM and enriched or not with TA312B2 were used as controls. Four replicates were made for each treatment. Wilt symptoms were assessed by the following scale: 0 = no disease; 1 = limited local symptoms; 2 = well developed symptoms; 3 = severe wilt or death. A weighted disease severity index was calculated. Data were arc sin transformed before ANOVA.
**Results**

Values of pH and salinity (Ec) were similar in LS-MSMC and MSMC. Higher content of NH$_4^+$-N and higher NH$_4^+/NO_3^-$ ratio were observed in LS-MSMC than in MSMC (Table 1). Numbers of TA 312B2 in MSMC and LS-MSMC significantly decreased from two days after enrichment (3x10$^5$ cfu/ml for both products) to 130 d after enrichment (5x10$^5$ and 2x10$^5$ CFUml$^{-1}$ for MSMC and LS-MSMC respectively). No statistical differences among the products were observed for microbial and chitinase activity (tab. 2). LS-MSMC with and without TA 312B2, showed the highest β-glucosidase activity.

**Table 1. Physical and chemical parameters of mature spent mushroom compost with and without calcium lignosulphonate**

<table>
<thead>
<tr>
<th>Product</th>
<th>pH</th>
<th>Ec mS/cm</th>
<th>Ash %</th>
<th>N org. mg/Kg</th>
<th>N tot mg/Kg</th>
<th>NO$_3^-$-N mg/Kg</th>
<th>NH$_4^+$-N mg/Kg</th>
<th>C tot mg/Kg</th>
<th>C/N</th>
<th>NH$_4^+$/NO$_3^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature spent mushroom compost</td>
<td>7.5</td>
<td>2.8</td>
<td>30.8</td>
<td>4155</td>
<td>11111</td>
<td>520</td>
<td>25</td>
<td>136000</td>
<td>12.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Mature spent mushroom compost + Ca-Lignosulphonate</td>
<td>7.5</td>
<td>2.7</td>
<td>30.9</td>
<td>4243</td>
<td>11034</td>
<td>512</td>
<td>45</td>
<td>137000</td>
<td>12.4</td>
<td>0.09</td>
</tr>
</tbody>
</table>

**Table 2. Microbial and enzymatic activity of the different organic products**

<table>
<thead>
<tr>
<th>Product</th>
<th>Microbial activity (µg FDA hydrolized /ml min)</th>
<th>Chitinase activity (µg PNP/ml h)</th>
<th>β-glucosidase activity (µg PNP/ml h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peat moss</td>
<td>0.95</td>
<td>39.82</td>
<td>57.99</td>
</tr>
<tr>
<td>Mature spent mushroom compost</td>
<td>1.91</td>
<td>70.05</td>
<td>130.17</td>
</tr>
<tr>
<td>Mature spent mushroom compost + TA 312B2</td>
<td>1.91</td>
<td>59.25</td>
<td>126.21</td>
</tr>
<tr>
<td>Mature spent mushroom compost + Ca-Lignosulphonate</td>
<td>1.79</td>
<td>73.72</td>
<td>177.62</td>
</tr>
<tr>
<td>Mature spent mushroom compost + Ca-Lignosulphonate+ TA 312B2</td>
<td>1.43</td>
<td>54.72</td>
<td>170.58</td>
</tr>
<tr>
<td><strong>LSD$_{0.05}$ (One-way ANOVA)</strong></td>
<td><strong>n.s</strong></td>
<td><strong>n.s</strong></td>
<td><strong>15.72</strong></td>
</tr>
</tbody>
</table>

**Table 3. Wilt disease severity of melon plants three weeks after the transplanting into the different products infected with Fusarium oxysporum f.sp. melonis**

<table>
<thead>
<tr>
<th>Product</th>
<th>Disease index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peat moss</td>
<td>25.7</td>
</tr>
<tr>
<td>Peat moss + TA 312B2</td>
<td>32.3</td>
</tr>
<tr>
<td>Mature spent mushroom compost</td>
<td>11.7</td>
</tr>
<tr>
<td>Mature spent mushroom compost + TA 312B2</td>
<td>14.4</td>
</tr>
<tr>
<td>Mature spent mushroom compost + Ca-lignosulphonate</td>
<td>9.4</td>
</tr>
<tr>
<td>Mature spent mushroom compost + Ca-lignosulphonate+ TA312B2</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>LSD$_{0.05}$ (One-way ANOVA)</strong></td>
<td><strong>8.6</strong></td>
</tr>
</tbody>
</table>
Melon plants grown in a pot mixture containing MSMC showed a significant reduction (40%) of the disease index value compared to the index value of plants developed in absence of MSMC (Table 3). The enrichment of MSMC with LS or TA 312B2 separately determined disease levels similar to that of not fortified MSMC. The addition of both LS and TA 312B2 significantly increased the suppressiveness of MSMC.

Discussion

Mature spent mushroom compost showed under the experimental conditions considered highly suppressive ability against the disease caused by *F. oxysporum* f. sp. *melonis*. The addition of Ca-Lignosulphonate and *T. atroviride* TA 312B2 separately did not affect the biocontrol ability of spent mushroom compost, whereas the enrichment with both drastically enhanced the compost suppressiveness. This could be explained by the increase of NH₄⁺-N level and β-glucosidase activity observed after fortification with Ca-Lignosulphonate, which might create in the spent mushroom compost an environment suitable for the suppressive activity of TA312B2. This is in part supported by the finding that high amount of NH₄⁺-N enhances saprotrophic activity and survival of *Trichoderma* in compost or soil (Montanari et al., 2004, Celar, 2003). In conclusion, mature spent mushroom compost seems to be a valid suppressive compost, however this result need to be confirmed in different pathosystems. The Ca-Lignosulphonate seems to be able to stimulate the suppressive ability of *Trichoderma* and this could be very interesting for improving *Trichoderma* fortified composts.

References

Can incomplete spatial coverage of control measures prevent invasion of fungal parasites?

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Abstract: We examine the invasion of a pathogenic fungus into populations of susceptibles. Combining epidemiological concepts with percolation theory we derive and test the following hypothesis: 1) fungal invasion into a population can be stopped by rendering a threshold proportion of the population immune for infection; 2) controlling infection at randomly selected sites introduces a shield which can prevent invasion of unprotected sites; 3) the rate of invasion reduces with increasing number of randomly protected sites. The significance of these findings is that the extent at which control strategies are applied spatially can be a critical component of disease management.

Key words: Rhizoctonia solani, invasion, percolation, thresholds for control

Introduction

Control of epidemics may aim to reduce inoculum density in a field, to reduce the spread of the pathogen, or to prevent infection upon contact by rendering the host resistant. Previously, we have shown that invasion of a pathogen can be prevented by only a small reduction in the ability of pathogens to spread (Bailey et al., 2000). For soil-borne fungal plant pathogens, such a reduction in spread can be achieved by altering soil physical properties (Otten et al., 2001) or by introducing biocontrol agents (Bailey & Gilligan, 1997). In this paper we consider if invasion of pathogens into a susceptible population can be achieved by rendering a certain fraction of hosts immune to infection. Whilst protection of each host in a plant population does prevent invasion, such complete spatial coverage is often not achieved, or is not economically viable. This raises the question what fraction of susceptible hosts needs to be protected to prevent an epidemic?

We explain how percolation theory derived from statistical physics can be used to predict biological invasion into a population. We subsequently test the theory for the spread of the fungal plant pathogen Rhizoctonia solani, and show that invasion can be stopped when a predicted critical fraction (c. 40%) of the population is made unavailable for colonisation. We discuss the broader consequences for the deployment of biocontrol.

The use of percolation theory to derive spatial thresholds to prevent invasion

We formulate our hypothesis starting with the simplest form of an epidemic. Consider a population of sites (e.g. a root, plant or a field) on a lattice (Figure 1) with a fraction \( p \) sites susceptible to infection, and a fraction \( (1-p) \) immune. Following the introduction of infection, an infected site can infect all non-immune nearest neighbours. The newly infected sites can subsequently infect their non-immune nearest neighbours, and so on, generating a patch of infected sites. Here we define a patch as a group of infected or susceptible sites that directly neighbour each other (Figure 1). Percolation theory deals with a variety of properties of such patches, including the size distribution and other geometric characteristics. These properties
are directly relevant for epidemics. The size distribution of the patches relates to the risk that an introduced infection evolves into a large epidemic, and the geometry of the patches relates to the dynamics of an epidemic, as small pockets of well connected susceptible sites are connected with each other by bottle necks of single susceptible sites (Havlin and Bunde 1991; Otten et al., 2004). What makes percolation theory particularly interesting is that it shows that these properties change abruptly with the number of immune sites in a population.

If the fraction of immune plants \((1-p)\) is large, the remaining susceptible sites form small patches or are isolated, and disease cannot invade. If on the other hand the number of immune sites is small, a large proportion of the susceptible sites form part of an infinite, well connected patch. If disease is introduced into such a population, and it falls within this infinite patch, it can invade. Percolation theory states that a threshold proportion exists where for the first time an invasive patch is formed. Below the threshold fraction of controlled sites disease is non-invasive, above the threshold disease can invade the population (Figure 1). Note that even when an invasive patch exists, several smaller patches also occur in the same population. If disease is introduced within one of the smaller patches, it would stop spreading. Consequently, a large degree of variability is expected in the efficacy of biocontrol even under homogeneous conditions.

**Figure 1.** Simulated examples of (a) non-invasive, or (b) invasive spread into a population in which a fraction 0.42 (a) or 0.4 (b) of sites is unavailable. At a critical fraction of removed sites the spread of a pathogen switches from invasive to non-invasive (c). Legend: invaded patch: black; available sites: grey; protected sites/unavailable: white. Note that as well as a large invasive patch (black in Figure b) several isolated smaller patches occur (grey sites), in which disease would stop spreading. A large degree of variability of the efficacy of control is therefore expected depending on where disease is introduced into the population.

**Practical example: thresholds to control invasion of the fungus R. solani.**
To test if this predicted threshold for invasion is observed in more variable biological systems, we quantified the spread of the fungal plant pathogen and saprotroph *Rhizoctonia solani* in replicated microcosms comprising discrete nutrient sites. Spread of a fungus through such a system can be seen as an analogue for an epidemic: fungal spread initiates from the primary colonisation of a nutrient site (cf. susceptible sites in a plant population), followed by secondary colonisation as mycelium spreads from a colonised (cf. infected) towards another susceptible site until the colonised site is depleted of nutrients (cf. death of the infected host). The advantage over the use of a plant system is that the susceptibility of sites is static in time,
i.e. their resistance does not increase with time as is often found with plants, and which may obscure the observation of a threshold.

**Design of the microcosms**

Each microcosm contained 160 sites on a square lattice, spaced 8 mm apart. A Fraction of 0, 10, 20, 30, 40, 50, or 60% of the sites was randomly selected to remain empty, therefore unavailable for colonisation as an analogue of successful control; the remaining sites contained a 3-mm diameter agar dot each, representing the susceptible fraction in the population. Each treatment was replicated 10 times, leading to 70 populations. The central site of each population was inoculated with a single hyphal strand (1 mm length) of *R. solani*, assessed daily, and the number and locations of colonised sites recorded. We define noninvasive spread as those replicates in which fungal spread initiated from a single site fails to progress to the edge of the population. By default the remaining replicates that did reach the edge are classified as invasive. We subsequently quantify the ability to invade as the proportion of replicate populations with invasive spread.

**Results**

The spread of *R. solani* through the populations follows closely the pattern of neighbouring sites (Figure 2a). For populations in which a significant fraction of sites had been removed, the spread appears as a line weaving through the population occasionally encountering a small patch of interconnected sites. The rate of spread in such a population was typically slow with short bursts of activity (Figure 2b). The fungus, however, was unable to colonise all sites showing that protecting/removal of susceptible sites shields a large proportion of the remaining susceptibles from invasion.

![Figure 2](image)

**Figure 2.** An example of spread of *R. solani* through a population of susceptibles (a). The dynamics of invasion (b) are typically slow with bursts of activity (circle). When a critical fraction of sites is removed from the population (c) the ability of *R. solani* to invade rapidly declines. Legend: ○: susceptible sites; ■: colonised sites; ▲: site of inoculation.

The central question we asked was how many sites need to be removed in a population to prevent invasion of a fungus. There is a clear transition from invasive to non-invasive spread with increasing number of removed sites (Figure 2c). Removal up to 30% of sites had almost no effect on the ability of *R. solani* to invade; removal of more than 60% of sites was sufficient to reduce the probability of invasion to less than 0.1. The experimentally observed
relationship was closely related to what would be expected for these relatively small microcosms.

**Ecological and epidemiological relevance of invasion thresholds**

There are major challenges facing epidemiologists in optimising the spatial scales at which to apply control measures with the increasing risk of fungicide resistance and the need to anticipate the occurrence of novel or re-emerging disease problems. Underlying many of these problems lies the concept of invasion: Will a disease invade, and how many sites need to be protected to prevent invasion? Combining theoretical arguments with empirical systems we demonstrated that if a critical proportion of susceptible sites is removed from a population, invasive spread of a pathogen can be prevented. The significance of the work is that it shows that spatially heterogeneous coverage of a control strategy can prevent biological invasion by treating not all but only a critical proportion of the susceptible population.

The epidemic system considered here is still somewhat removed from natural systems. However, theoretical developments are ongoing from which similar hypotheses for more realistic systems can be derived. As percolation theory can be applied at a range of scales, we conjecture that the thresholds for spatial coverage of control measures may apply to a range of epidemiological systems. These include the invasion through heterogeneous environments with plant communities and agricultural crops occurring in mosaics of many species in patterns ranging from the scale of fields (e.g. fields with resistant varieties or fields in which specific control measures have been applied), to within crop diversity. Reducing the area at which control is applied whilst still preventing invasion can be economically beneficial, and may also reduce the risk of increasing resistance. In other situations, e.g. the deployment of biological control agents on root systems, it is difficult to obtain full spatial coverage. We suggest that the theoretical approaches followed here provide a fertile basis for the analysis of epidemiological systems, leading to relatively simple estimates for the coverage of control required to prevent invasion.

**Acknowledgements**

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**References**


Interaction between rhizoplane bacteria and a phytopathogenic Peronosporomycete Aphanomyces cochlioides in relation to the suppression of damping-off disease in sugar beet and spinach

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Abstract: We investigated the modes of root colonization and antibiosis of Lysobacter sp. strain SB-K88 and other rhizoplane bacteria of spinach and sugar beet antagonizing a Peronosporomycete pathogen, Aphanomyces cochlioides. The SB-K88 has long brush-like fimbriae at one pole of the sessile bacterial rod. Scanning electron microscope analysis of two weeks old seedlings of sugar beet and spinach upon inoculation of seeds revealed that SB-K88 densely colonized the root and cotyledon surfaces in a perpendicular fashion using polar fimbriae and developed biofilm-like structures on roots covered by root mucigel. Seed treated with either SB-K88 or its culture filtrate significantly suppressed A. cochlioides damping-off in sugar beet and spinach. In dual culture assay, SB-K88 and other rhizoplane bacteria caused excessive branching, swelling and loss of radial growth in the approaching hyphae of A. cochlioides. Remarkable ultrastructural alterations in the affected hyphae were observed under transmission electron microscope. Interestingly, zoospores of A. cochlioides, were rendered immotile within 1 min of exposure to cell suspension or cell free culture supernatant or EtOAc extracts or pure xanthobaccin A (1) isolated from SB-K88, and subsequent lysis occurred within 30 min. Our observations provide the convincing evidence that Lysobacter sp. exerts a direct inhibitory effect on A. cochlioides and suppresses damping-off in sugar beet and spinach through a combination of antibiosis and high root colonization.

Key words: Lysobacter sp., root colonization, zoospore lysis, Aphanomyces cochlioides, sugar beet

Introduction

The members of Peronosporomycetes (Oomycetes in the old classification) genera such as Aphanomyces, Phytophthora, and Pythium are the devastating pathogens of many economically important crops. Root rot and damping-off caused by Aphanomyces cochlioides, Pythium ultimum and Rhizoctonia solani are the serious diseases of sugar beet all over the world. A. cochlioides also infects spinach, feather cockscomb and some other members of Chenopodiaceae and Amaranthaceae. The biflagellated zoospores of A. cochlioides liberated from the mycelium locate host roots guided by a host-specific flavonoid signal, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone) released from the roots, after which they undergo a series of morphological changes before penetrating the host tissues (Islam et al., 2003). Disruption of any of these homing events eliminates the potential for pathogenesis. Homma et al. (1993) isolated a number of antagonistic bacterial strains against Pythium sp. from the rhizoplane of sugar beet where one of the isolates, Lysobacter sp. strain SB-K88 (tentatively identified as Xanthomonas or Stenotrophomonas sp.) is a promising control agent of root rot and damping-off diseases in sugar beet through production of a major metabolite, xanthobaccin A (1) (Nakayama et al., 1999). However, the effect of xanthobaccin A against zoospores (infecting organs) of A. cochlioides, but the ability of colonization of SB-K88 on
the host roots have not yet been investigated. Therefore, the objectives of the present work were to i) investigate the modes of attachment and patterns of bacterial colonization of host root upon inoculation to the seed; ii) test the effects of live bacteria and their secondary metabolites on the approaching hyphae, and survival and homing events of zoospores; iii) examine the host range of SB-K88 with respect to root colonization; and iv) search other potential rhizoplane biocontrol bacteria against *A. cochlioides*.

**Materials and methods**

*Culture condition and extraction*

*Lysobacter* sp. strain SB-K88 was cultivated in liquid medium at 25°C for 15 days by shaking. The culture suspension was centrifuged and the supernatant was freeze-dried. The residue was subsequently extracted with EtOAc and MeOH and then concentrated *in vacuo*.

*Zoospore production and bioassay*

*A. cochlioides* (AC-5) was cultured on corn meal agar on a glass Petri dish (9 cm i.d.), and the zoospores were produced as reported before (Islam et al., 2003). Zoospore bioassay of live bacteria, freeze-dried materials, EtOAc extracts, water solubles, and pure xanthobaccin A (1) (Nakayama et al., 1999) were done by homogeneous solution method (Islam et al., 2002). Seed pelleting, zoospore inoculation and disease intensity

Sterilized seeds (Daigaku-Noen, Tokyo) of sugar beet and spinach were pelleted with SB-K88 (c. 10^7 cfu/seed) (Nakayama et al., 1999), and grown in 0.2% gellan gum containing 1/5 Hogland’s S medium in test tubes or in sterilized soils in 36-cells small plastic pack. On day 12, each seedling was inoculated with 1 ml of an *A. cochlioides* zoospore suspension containing 1´10^4, 1´10^3 or 1´10^2 zoospores. Data for the percentage of healthy seedlings were recorded after 2 weeks of zoospore inoculation. Each treatment was replicated twice.

*SEM and TEM observations*

Colonization of bacteria to the roots grown in gellan gum or soils, and zoospore lytic activity of xanthobaccin A (1) were observed by scanning electron microscopy (SEM) on day 14 of cultivation as described previously (Islam et al., 2003). Ultrastructural changes of approaching hyphae of AC-5 toward bacterial colony of SB-K88 in the PDA medium were observed by transmission electron microscopy (TEM).

**Results and discussion**

*Characteristic attachment and colonization of Lysobacter sp. SB-K88*

TEM observation revealed that Lysobacter sp. strain SB-K88 has very long (~6 µm) brush-like fragile fimbriae on one pole of the sessile bacterial rod (~1 µm) (Figure 1a). Scanning electron microscopy revealed that upon seed inoculation, SB-K88 highly colonized and attached perpendicularly on the roots of both hosts (spinach and sugar beet) and non-hosts (tomato, *Amaranthus gangeticus* and *Arabidopsis thaliana*) of *A. cochlioides* when plant seedlings were grown in the gnotobiotic systems containing gellan gum or soil media (Figure 1b-g). In the case of host roots, i) SB-K88 densely colonized the root and cotyledon surfaces in a perpendicular fashion using polar fimbriae (Figures 1b, d); ii) a stable biofilm-like structure covered by root mucigel (Figure 1c); and iii) micro-colonies localized mainly at the junction between primary and secondary roots with bacterial numbers declining from the top to bottom of the root. However, no root mucigel covered stable biofilm was observed in the case of non-host plants. The distinct features of *Lysobacter* sp. colonization to plants were the perpendicular attachment and the development of a stable biofilm on the root. Similar
phenomenon was observed in the opportunistic pathogen *Pseudomonas aeruginosa* strain PA14 to the surface of human respiratory epithelial cells and also to plants (Plotnikova et al., 2000). Effective colonization of both foliar and subterranean plant parts has also been observed in *L. enzymogenes* (Sullivan et al., 2003).

**Motility inhibition and lysis of zoospores**

In addition to mycelial growth inhibition, the zoospores of *A. cochlioides*, were rendered immotile (100%) within 1 minute of exposure to cell suspension (109 bacteria/ml) or cell culture supernatant (100 µg/ml) of the SB-K88, and subsequent lysis (~40%) occurred within 30 minutes. Crude EtOAc extract (MIC 10 µg/ml) of the culture supernatant or pure xanthobaccin A (1) (MIC 0.01 µg/ml) also caused identical motility inhibition (100%) followed by lysis of zoospores in a dose dependent manner (Figure 1k). Chemical fractionation of crude extracts revealed that xanthobaccin A (1) (Figure 1) produced by the SB-K88 was primarily responsible for mycelial growth inhibition and lysis of zoospores. Production of zoospores from the *A. cochlioides* mycelia was remarkably reduced (~50%) in the presence of SB-K88 at a dose of 10^7 cells/ml. Lytic activity against other microorganisms is a generic characteristic of *Lysobacter* spp. (Christensen & Cook, 1978). However, motility inhibition followed by lysis of zoospores shown in this report has not been claimed so far.

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![Figure 1](image_url)

**Figure 1.** Colonization of *Lysobacter* sp. (SB-K88) to plant surfaces, and antagonistic activities towards *Aphanomyces cochlioides*

- a. big fimbriae at one end of bacterium; b and d. colonization by perpendicular attachment to sugar beet root (b) and cotyledon (d); c. bacterial biofilm covered by root mucigel of sugar beet; e. colonization of the tomato root; f and g. colonization by perpendicular attachment (insets) to *Arabidopsis thaliana* leaf (f) and root (g); h and k. normal cystospores (h) (control) and lysed zoospores (k) by xanthobaccin A (1) at 1 ppm; i. hyphal growth inhibition by SB-K88 (4 days); j and n. ultrastructure of control (j) and affected (n) hyphae; l and m. control (m) and affected hyphae (l) approaching towards SB-K88; o and p. damping-off suppression in spinach (o) and sugar beet (p). Tachigaren: a commercial fungicide.
Morphology and ultrastructural alterations in approaching hyphae
Excessive branching, irregular swelling and loss of apical growth in the *A. cochlioides* hyphae approached to the bacterial colony (dual culture in PDA) were observed under light microscopy (Figure 1l). Remarkable ultrastructural alterations including considerable thickening of hyphal cell walls, extensive vacuolization, accumulations of lipid bodies and degeneration of the hyphal cytoplasm were observed in the approaching hyphae by transmission electron microscopy (Figure 1n).

Disease suppression activity
Seeds treated with either SB-K88 (10^8 cells/seed) or its cell free culture supernatant (100 µg/g soil) significantly suppressed damping-off in both sugar beet (65% healthy plants) (Figure 1p) and spinach (85% healthy plants) (Figure 1o) when seedlings were artificially infested with the zoospores (10^3 zoospores/seedling) of *A. cochlioides*. Our observations provide the convincing evidence that *Lysobacter* sp. exerts a direct inhibitory effect on *A. cochlioides* and suppresses damping-off disease in sugar beet and spinach through a combination of antibiosis and high root colonization.

Other antagonistic rhizoplane bacteria
We screened 150 rhizoplane bacterial isolates from the host and non-host plants, and evaluated their antagonistic activity towards the growth of *A. cochlioides*. Some of the strains (~5%) of *Pseudomonas* spp., *Stenotrophomonas* spp., *Klebsiella* spp., *Delftia* sp. and *Bacillus* sp. exhibited characteristic hyphal morphological alterations: excessive branching, spiral growth, longer and pointed tip formation, and cytoplasmic disintegration on PDA medium, all of which was highly correlated with *in vivo* disease suppression activity of the respective bacteria in sugar beet and spinach. These results suggest that hyphal morphological alterations could be considered as a novel criterion for screening potent biocontrol rhizobacteria.

Acknowledgements
We are thankful to Professor R. Yokosawa, Health Science University of Hokkaido for his kind gift of *A. cochlioides* (AC-5). The financial support and a Postdoctoral Fellowship (to M.T.I) from the Japan Society for the Promotion of Science (JSPS) are also very much appreciated.

References

Studies on efficacy and mode of action of rhizosphere bacteria against *Phytophthora* spp. in strawberry

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**Abstract:** After screening of several rhizosphere bacteria against the soilborne pathogens of red core and crown rot disease of strawberry *Phytophthora fragariae var. fragariae* and *Phytophthora cactorum* under *in vitro* conditions, three of the most active isolates such as *Raoultella terrigena* (G-584), *Bacillus amyloliquefaciens* (G-V1) and *Pseudomonas fluorescens* (2R1-7) were selected for further studies under *in vivo* conditions. In greenhouse and field experiments, the three isolates were tested against both *Phytophthora* diseases under artificial infested soil conditions. Root dip treatment with these bacterial antagonists produced a control effect on both fungal diseases up to 55% and were nearly comparable with the chemical fungicide Aliette. First studies on the mode of action of antagonists showed differences in enzymatic reaction.

**Key words:** Biocontrol, Rhizobacteria, *Phytophthora fragariae* var. *fragariae*, *Phytophthora cactorum*

**Introduction**

Strawberry is one of the important berry cultures in Germany, which is attacked by several viral, bacterial and fungal diseases. Of the fungi attacking strawberries two of the most important pathogens *Phytophthora fragariae var. fragariae* and *Phytophthora cactorum*, which are the cause of red core and crown rot disease of strawberry, can cause substantial economical damage in strawberry production (Seemüller, 1998). In areas, where *P. fragariae* var. *fragariae* and *P. cactorum* are present, cultivation of strawberry depends directly on the use of chemicals. The number of chemical fungicides available for the control of both *Phytophthora* pathogens are limited. In most countries two different fungicides viz. Ridomil with Metalaxyl and Aliette with Aluminium-Fosetyl as active substance are available against these pathogens. In some fields where Metalaxyl was used for years, resistant strains of *P. fragariae* var. *fragariae* have been found (Nickerson, 1998; Seemüller et al., 1989). The problems associated with the use of agrochemicals have promoted research in the field of biological control of plant diseases as an alternative method of control. Hence in this study, an attempt has been aimed to control both *Phytophthora* diseases by using different antagonistic bacteria.

**Material and methods**

*Bacteria and pathogens*

After a screening of more than 100 bacterial isolates out of the rhizosphere of fruit orchards in dual culture test, three with the best inhibition effect against the two *Phytophthora* pathogens, were further used for *in vivo* experiments. The following bacterial isolates were used: *Raoultella terrigena* strain G-584, *Bacillus amyloliquefaciens* strain G-V1 and *Pseudomonas*...
fluorescens strain 2R1-7. Isolates G-V1, G-584 were formulated by the company E-nema, Raisdorf. The 2R1-7 isolate was prepared by growing in Tryptic Soy Broth for 24 h at 26°C, cells were harvested by centrifugation and diluted to an optical density of 0.20 at 660 nm; $10^8$ to $10^9$ cfu/ml were used for the experiments. Inocula of *P. fragariae* and *P. cactorum* were cultured in vermiculite media (composed of seeds of rye) for four weeks at 18°C and 3% of inoculum used for artificial infestation of soil.

**Plant material and greenhouse experiments**

Tests were performed with strawberry (*Fragaria X ananassa* Duchesne) plants of cv. Elsanta (Frigo). This variety of strawberry is highly susceptible to red core and crown rot disease. Roots of strawberry plants were dipped for 15 min into the bacterial suspension in order to have direct exposure of the roots to the antagonistic bacteria. Roots of control plants were either dipped in tap water or 0.5% Aliette. Plants were then transferred to 14 cm plastic pots with artificially infested soil using 3% of inoculum. The experiment was arranged in two separate blocks, each with 10 pots per treatment. Ninety days after planting, roots were carefully washed and symptoms were scored using a 0-5 scale and disease index calculated. Statistical significance was tested using Duncan's Multiple Range Test ($P \leq 0.05$).

**Field experiments**

Field experiments were carried out under artificially infested soil conditions (3% of inoculum was applied to each planting hole). Plants were treated with bacteria by root dip as described above. Plants were planted in four rows with at least 40 plants per treatment (arranged in randomised design). Harvesting of plants (12 weeks after planting), rating of disease severity, computerization of disease index and statistical significance were all made as described before.

**Enzyme assays**

Two different methods were used. Firstly, Microplate assay according to Wirth & Wolf (1992): bacterial culture filtrates, substrates and buffer were kept in a water bath at 37°C for 1h. The reaction of 4 enzymes was terminated by adding 1N HCl then the sample was centrifuged, supernatant was measured at 595 nm for enzyme activity. Secondly API ZYM (Head & Ratnam, 1988) was employed, a semi quantitative micromethod consisting of 20 micro cupules, 19 of which contain dehydrated chromogenic substrate for detecting 19 performed enzyme activities. The test strips are inoculated and incubated aerobically at 37°C for 4 h, and the two reagents are added to develop the chromogenic substrates. The resultant colorimetric reactions are indicative of the degree of enzyme activity and they are compared with the control well and a colour chart.

**Results and discussion**

In greenhouse experiment, rhizobacteria showed different levels of biocontrol activity towards both pathogens (Table 1). Highest control percentage of 54.7% against red core was achieved from isolate 2 R1-7 of *P. fluorescens*, whereas against crown rot isolate G-584 of *Raoultella terrigena* showed the best effect with 55.0%. This was nearly comparable with chemical control Aliette (64.7%).

The tested bacterial isolates also gave a considerable degree of protection against both pathogens under field conditions. In a field experiment with artificially infested soil (Table 2), the isolate 2 R1-7 of *P. fluorescens* showed also here highest efficacy up to 50.6% followed by G-584 and G-V1 against red core disease but a lower degree of control against crown rot between 32.7 to 37.9%. First results on the mode of action of the three antagonistic strains are presented in Table 3, where the enzyme profile of the isolates of *R. terrigena*, *B. amyloliquefaciens* and *P. fluorescens* were recorded according to two different methods of
enzyme assay. Highest number of enzyme activities were found from \textit{B. amyloliquefaciens} (cellulase, glucanase, alkaline phosphatase, esterase (C4) and esterase lipase (C8), followed by \textit{R. terrigena} (glucanase, alkaline phosphatase, leucine arylamidase and acid phosphatase) and \textit{P. fluorescens} showed only chitinase activity. If these test findings can be related to the antagonistic activity of the three biocontrol agents, must be clarified in further studies. In general these enzymes have been reported as part of biocontrol activity of bacterial antagonists.

Table 1. Influence of Rhizobacteria on disease index of red core and crown rot of strawberry under artificial infested soil conditions in the greenhouse

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Red core Disease index (%)</th>
<th>Control (%)</th>
<th>Crown rot Disease index (%)</th>
<th>Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42 a</td>
<td>51 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-584</td>
<td>26 bc</td>
<td>38.1</td>
<td>23 b</td>
<td>55.0</td>
</tr>
<tr>
<td>G-V1</td>
<td>27 bc</td>
<td>35.7</td>
<td>26 b</td>
<td>49.0</td>
</tr>
<tr>
<td>2 R1-7</td>
<td>19 c</td>
<td>54.7</td>
<td>26 b</td>
<td>49.0</td>
</tr>
<tr>
<td>Aliette</td>
<td>16 c</td>
<td>61.9</td>
<td>18 b</td>
<td>64.7</td>
</tr>
</tbody>
</table>

90 days after treatment, n=20.

Table 2. Influence of Rhizobacteria on disease index of red core and crown rot of strawberry under artificial infested soil conditions in the field (June 2003)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Red core Disease index (%)</th>
<th>Control (%)</th>
<th>Crown rot Disease index (%)</th>
<th>Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected Control</td>
<td>37.5 a</td>
<td>29.0 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-584</td>
<td>23.0 b</td>
<td>38.6</td>
<td>18.0 bc</td>
<td>37.9</td>
</tr>
<tr>
<td>G-V1</td>
<td>22.5 b</td>
<td>40.0</td>
<td>19.5 bc</td>
<td>32.7</td>
</tr>
<tr>
<td>2 R1-7</td>
<td>18.5 b</td>
<td>50.6</td>
<td>19.0 bc</td>
<td>34.4</td>
</tr>
<tr>
<td>Aliette</td>
<td>18.0 b</td>
<td>52.0</td>
<td>14.5 c</td>
<td>50.0</td>
</tr>
<tr>
<td>Untreated control</td>
<td>6.5 c</td>
<td>7.0 d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

90 days after treatment, n=40.

The bacterial strains tested for biocontrol activity towards \textit{Phytophthora} spp. have been previously shown to have biocontrol activity against different pathogens on a variety of different crops except the bacterial isolate \textit{Raoultella terrigena} (of our knowledge). In our studies, all strains showed different level of protection against both \textit{Phytophthora} spp. Variation in antagonistic activity of rhizobacteria with time and in different systems is a known phenomenon (Weller, 1988) and still difficult to explain. Biotic factors such as concurrence in root colonization with soil specific microbial communities or infection by other soil pathogens may play a role. Abiotic factors such as soil type, moisture and temperature as well as variations in application technology also may be of importance (Deacon, 1991). Further studies under naturally infested soil condition are in progress to elucidate these open questions.
Table 3. Determination of enzymes from bacterial antagonists

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Raoultella terrigena</th>
<th>Bacillus amyloliquefaciens</th>
<th>Pseudomonas fluorescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro plate assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulase</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Glucanase</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Chitinase</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Test kit api ZYM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Esterase (C 4)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esterase lipase (C 8)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Enzyme evaluation methods Micro-plate assay(4) & Test kit API ZYM (19).

Acknowledgements

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References


Eradication of plant pathogens and pests from composting wastes and their use in disease suppression

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Abstract: The objectives of this work were to determine the conditions required to eradicate a number of organisms from composting waste and the potential of different composts to suppress soil-borne diseases. Small-scale flask experiments were conducted to determine the effect of composting conditions on various plant pathogens (Fusarium oxysporum, Pythium ultimum, Olpidium brassicae, Rhizoctonia solani, Plasmodiophora brassicae, Xanthomonas campestris and Tobacco mosaic virus) and pest species (Delia antiqua and Psila rosae). The organisms were placed in the centre of horticultural wastes in flasks and incubated for up to seven days at temperatures ranging from 30-90°C. Following incubation, the viability of the organisms was determined. Most of the organisms examined were either completely destroyed or their populations significantly reduced when incubated above 50°C for seven days. A temperature of 80°C was required to destroy the Tobamovirus, Tobacco mosaic virus. Glasshouse pot bioassays were used to determine the effect of compost incorporation on suppression of root rots (Phytophthora nicotianae and Rhizoctonia solani) on tomatoes and club root (Plasmodiophora brassicae) on Chinese cabbage. The incidence of both the root rots (P. nicotianae and R. solani) and club root was significantly reduced in the presence of the composted wastes. The level of suppression varied with the inoculum dose, type and rate of compost incorporation.

Key words: composted wastes; disease suppression; soil-borne plant pathogens

Introduction

Currently, landfill is the most popular route for disposal of vegetable wastes in Europe. With the decrease in the availability of landfill sites, and the need to reduce the quantity of biodegradable waste disposed in this way in accordance with the EU Landfill Directive, an alternative to landfill disposal is required. One possible option is to compost the waste, to eradicate any pests and pathogens present, and then incorporate the composted waste into soil or peat for subsequent crop production. Soil incorporation of composted wastes is known to improve various soil properties. In addition, composted wastes have been shown to suppress various soil-borne pathogens and hence disposal of wastes in this way offers the possibility of sustainable disease control. The objectives of this work were to determine the conditions required to eradicate a number of organisms from composting waste and the potential of different composts to suppress soil-borne diseases.
Materials and methods

Eradication of pathogens and pests during composting

Small-scale flask experiments were conducted to determine the effect of composting conditions on various plant pathogens (Fusarium oxysporum, Pythium ultimum, Olpidium brassicae, Rhizoctonia solani, Plasmodiophora brassicae, Xanthomonas campestris and Tobacco mosaic virus) and pest species (Delia antiqua and Psila rosae). The organisms were placed in the centre of horticultural wastes (onion waste, green waste and/or spent mushroom compost (SMC)) in flasks and incubated in thermostatically controlled waterbaths for up to seven days at temperatures ranging from 30-90°C. Following incubation, the viability of the organisms was determined using selective media (F. oxysporum, P. ultimum, Rhizoctonia solani and X. campestris), inoculation of young plants (O. brassicae, P. brassicae and TMV) or visual inspection (D. antiqua and P. rosae).

Disease suppression bioassays with composted wastes

Glasshouse pot bioassays were used to determine the effect of compost incorporation on suppression of root rots (Phytophthora nicotianae and Rhizoctonia solani) on tomatoes and club root (Plasmodiophora brassicae) on Chinese cabbage.

Phytophthora nicotianae - tomato

Finnpeat inoculated with P. nicotianae was incorporated at a 1% (v/v) rate into Finnpeat containing 20% (v/v) of various waste types (SMC from the UK, France and Greece, green waste from Ireland and the Netherlands, and onion waste compost). Tomato seed (variety Aromata) was sown in the infested peat-waste mixtures and incubated in a glasshouse (14°C, 16 h day, 12°C, 8 h night). Germination and incidence of disease (scored as dead or dying plants) was recorded weekly for up to 8 weeks. There were 3 replicates of 5 plants for each treatment.

Rhizoctonia solani - tomato

French green waste compost and French SMC were incorporated into heat treated soil from Dijon, France at 10% and 30% (v/v) rates. The soil-compost mixtures were infested with R. solani (strain 1556) grown on barley grains at a rate of 0.5% (w/v, 2.5 g of inoculum per 0.5 l of soil). Tomato seed (variety Montfavet) was sown in the infested soil-compost mixtures and incubated in a growth chamber (25°C, 16 h day; 23°C, 8 h night) for 2 weeks. There were 3 replicates of 8 plants per treatment. At harvest, the tomato plants were scored for disease severity (0 = healthy plants; 1 = small necrosis on root; 2 = several necroses of small size; 3 = necrosis on half of the crown or the primary root; 4 = deep necrosis on the entire crown or the circumference of the primary root; 5 = dead).

Plasmodiophora brassicae - Chinese cabbage

French green waste compost and French SMC were incorporated into heat treated soil at two rates as described above. P. brassicae spores were added to the soil-compost mixtures to produce two levels of infestation (10^4 spores/ml and 10^5 spores/ml). Chinese cabbage seed (susceptible variety Granat) was sown in the infested soil-compost mixtures and incubated in a growth chamber (22°C, 16 h day; 20°C, 8 h night) for 6 weeks. There were 3 replicates of 5 plants per treatment. At harvest, the Chinese cabbage plants were removed from the growing media and their roots assessed for symptoms of club-root (0 = no gall; 1 = 1-2 galls; 2 = many galls but roots still healthy; 3 = entire root system attacked).
Results

Eradication of pathogens and pests during composting

Most of the organisms examined were either completely destroyed or their populations significantly reduced when incubated above 50°C for 7 d (Table 1). The incubation period was found to be important in determining eradication. *R. solani* and *P. ultimum* were eradicated at 40°C after 7 d incubation but were still viable after 3 d incubation. As temperature was increased the incubation period required to eradicate these organisms decreased. The moisture content of the waste was also found to influence pathogen survival. Eradication of *P. brassicae* after 1 or 3 d incubation was more efficient when the moisture content of the waste (SMC) was above 50%. A temperature of 80°C was required to destroy the *Tobamovirus*, Tobacco mosaic virus.

Table 1. Effect of incubating various pathogens and pests in onion waste, green waste and/or SMC for 7 d at different temperatures.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Waste</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Survival</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em> f.sp. pisi</td>
<td>onion</td>
<td>40</td>
</tr>
<tr>
<td><em>F. oxy. f.sp. narcissi</em></td>
<td>onion</td>
<td>45</td>
</tr>
<tr>
<td><em>F. oxy. f.sp. lycopersici</em></td>
<td>onion, green</td>
<td>46</td>
</tr>
<tr>
<td><em>F. oxy. f.sp. radicis lycopersici</em></td>
<td>onion, green</td>
<td>40</td>
</tr>
<tr>
<td><em>Pythium ultimum</em></td>
<td>onion, green</td>
<td>18</td>
</tr>
<tr>
<td><em>Olpidium brassicae</em></td>
<td>onion</td>
<td>18</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em></td>
<td>onion, SMC</td>
<td>18</td>
</tr>
<tr>
<td><em>Plasmodiophora brassicae</em></td>
<td>SMC, green</td>
<td>40</td>
</tr>
<tr>
<td><em>Xanthomonas campestris</em></td>
<td>onion, green</td>
<td>18</td>
</tr>
<tr>
<td>Tobacco mosaic virus</td>
<td>onion</td>
<td>70</td>
</tr>
<tr>
<td><em>Delia antiqua</em></td>
<td>onion</td>
<td>18</td>
</tr>
<tr>
<td><em>Psila rosae</em></td>
<td>onion</td>
<td>18</td>
</tr>
</tbody>
</table>

* Lowest incubated temperature treatment tested.

* Providing moisture content is above 50%.

Disease suppression bioassays with composted wastes

*Phytophthora nicotianae*-tomato

There was no significant difference between the germination of the tomato seed in the control (Finnepeat only) and waste amended treatments. The presence of the UK and French SMC, the Irish and Dutch green wastes, and the composted onion waste significantly reduced the incidence of disease compared to the control by more than 40% (Figure 1).

*Rhizoctonia solani*-tomato

The green waste compost incorporation reduced disease severity proportional to the rate of compost added to the soil. The disease index was reduced from 20.3 in the control to 13.5 and 4.7 in the presence of 10% and 30% green waste compost respectively (Figure 2). The 10% rate of SMC reduced the disease index to 6.7 whereas the 30% rate had no effect on disease suppression (Figure 2).
Figure 1. Effect of incorporation (20% (v/v)) of different waste types (SMC from the UK, France and Greece, green waste from Ireland and the Netherlands, and onion waste compost) into Finnpeat on the suppression of Phytophthora nicotianae on tomato after 8 weeks.

Figure 2. Effect of green waste and SMC incorporation (10 and 30% (v/v)) into soil on the suppression of Rhizoctonia solani on tomato

Figure 3. Effect of green waste and SMC incorporation (10 and 30% (v/v)) into soil on the suppression of Plasmodiophora brassicae on cabbage. *P. brassicae* inoculum: (a) $10^5$ and (b) $10^5$ spores/ml
**Plasmodiophora brassicae**-Chinese cabbage

At the lower inoculum concentration of $10^4$ spores/ml, both composts provided complete disease suppression when introduced at a rate of 30% (Figure 3a). The 10% rate of the composts also had an effect on disease severity with the disease index reduced from 73 in the control to 48 and 47 in the soil-compost mixtures with green waste and SMC respectively (Figure 3a). At the higher inoculum concentration of $10^5$ spores/ml, the 30% rate of the composts induced almost complete disease suppression (Figure 3b). In contrast, the 10% rate of the composts had no effect on disease suppression (Figure 3b).

**Discussion**

Approximately 60 million tonnes of organic waste is produced in the EU annually (Slater & Frederickson, 2001). Currently, over 50 million tonnes is disposed of in landfill. The EU Landfill Directive requires member states to divert more organic waste from landfill. Alternative routes for the disposal of agricultural and horticultural wastes will therefore become increasingly important. Whilst the suppressive effect of various composted wastes has been demonstrated for numerous plant-pathogen interactions (Noble & Coventry, 2004), there is a need to assure potential end-users that the composted waste will not introduce pests or pathogens. The conditions required for the eradication of plant pathogens and nematodes during composting have recently been reviewed (Noble & Roberts, 2004). It was reported that 33 fungal pathogens, 7 bacterial pathogens, 9 nematodes and 3 plant viruses were destroyed by temperatures between 64-70°C held for up to 21 d (Noble & Roberts, 2004). In this study, all the organisms examined, with the exception of TMV, were eradicated after seven days incubation at 50°C. These temperatures are routinely achieved during composting. There is, therefore, a need to compost under specified conditions of time and temperature to ensure unwanted organisms present in the wastes are destroyed and will not subsequently infect the growing media or land to which they are applied (Bollen et al., 1989; Coventry et al., 2002). This should alleviate the concerns of end-users with respect to compost hygiene and provide a sustainable method of controlling various plant diseases and disposing of organic waste.

**Acknowledgements**

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**References**


Multi-target biocontrol efficacy of Clonostachys rosea IK726

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Abstract: Clonostachys rosea, IK726 has proved to be effective in controlling seed- and soil-borne diseases under diverse environmental conditions. Moreover, the antagonist can thrive and survive in very different niches such as natural soil, seed, and root and leaf surfaces. A gfp-transformant of IK726 has been a very helpful tool in ecological studies.

Key words: Fusarium culmorum, Bipolaris sorokiniana, Alternaria radicina, A. dauci, Pythium tracheiphilum, gfp-transformant

Introduction

The filamentous fungus Clonostachys rosea (syn. Gliocladium roseum: teleomorph Bionectria ochroleuca) has emerged as an effective and versatile antagonist in different crops (e.g. reviewed by Sutton et al., 1997). Clonostachys isolates also turned out to be the most effective fungal candidates against seed borne cereal diseases in a Nordic screening program, where the candidates were isolated from and evaluated on cereals (Knudsen et al., 1997). The biocontrol efficacy of one of the isolates, C. rosea IK726, against seed borne Fusarium culmorum and Bipolaris sorokiniana was confirmed in field experiments (Knudsen et al., 1995a; Jensen et al., 2000). The same in planta screening approach was used for selection of antagonists to control Alternaria radicina and A. dauci on carrot seeds, and again Clonostachys isolates were the most effective fungal antagonists (Jensen et al., 2004). However, C. rosea IK726, isolated from barley roots, was as effective as Clonostachys candidates selected from carrot material. It is therefore suggested that Clonostachys spp. and in particular C. rosea IK726 have multi-target effects. Further examples and description of the ecological diversity of the fungus are given below.

Combining IK726 application with physiological seed treatment methods under different temperatures

Besides using traditional ways of applying BCAs, we investigate the feasibility of integrating C. rosea IK726 application with physiological seed treatment methods characterized by high seed moisture contents (MC).

Seed priming of carrot seeds enhances field establishment especially of early sown crops. However, if seeds to be primed are infected by fungal pathogens, these may multiply during the priming process and counteract the positive effects of priming. Jensen et al. (2004) demonstrated that pathogenic Alternaria species increase significantly during 14 days hydro-priming (38-40% seed MC. 15°C) causing strong reduction in final stand of healthy seedlings compared to that of seedlings from non-primed seeds. Further studies show that the occurrence of several saprophytic fungal species also increases during priming (Figure 1). In contrast, addition of IK726 during priming (bio-priming) almost eradicates seed-borne
*Alternaria radicina* and *A. dauci* and a range of saprophytic fungi as well (Figure 1). Significant improvement of seedling establishment by IK726 biopriming has been demonstrated both in growth chamber (Jensen et al., 2004) and field experiments ( Birgit Jensen unpubl.). Apart from IK726 we have also tested the performance of other *Clonostachys* spp. and a *Plectosporium* isolate in bio-priming. Especially the *Clonostachys* spp. seem well adapted to the high humidity environment during priming and almost completely out-competes the indigenous fungal microflora (Figure 1).

Storage of seed may be associated with reduction of seed germination and establishment, due to seed borne pathogenic and saprophytic fungi. Thus, acorns are recalcitrant seeds, which must be stored at high moisture content and at low temperature, which are conditions that also favour growth of specific pathogenic and saprophytic fungi. We have studied the capacity of *C. rosea* IK726 to counteract such problems, and we have demonstrated that it can establish on acorns during several months of cold storage (-1°C). This positively affected acorn survival and reduced growth of pathogenic fungi such as *Ciboria batschiana* and of other cold storage fungi (*Acremoniella atra, Fusarium* spp. and *Alternaria* spp.) (Knudsen et al., 2004).

Breaking of dormancy of *Abies* seeds involves low temperature and high moisture conditions (5°C, 33% MC) during the stratification procedure. We currently investigate the possibility of controlling seed and soil borne fungi that reduce seed vigour, germination and plant establishment through activity during stratification and after sowing. *Abies nordmanniana* seed germination capacity and the frequency of healthy seedlings was found to improve significantly, when IK726 was applied to seed (clay formulation, 4 x 10⁹ cfu/g clay, applied at 0.005g/g seed) prior to stratification as compared to untreated seed. Such pre-stratification seed treatment with IK726 also significantly improved seedling emergence in sowings on land naturally infested with *Pythium* spp. and *Fusarium* spp. as compared to post-stratification seed treatment (Inge M.B. Knudsen, unpublished).

Figure 1. Effect of biopriming on occurrence of fungi on carrot seeds (1755: *Plectosporium tabacinum*; 1871: *Clonostachys rosea*; 1878: *Clonostachys catenulatum*; 1889: *Clonostachys solani* and IK726d11: *Clonostachys rosea*, gfp-transformant of IK726).
Biological control of soil borne Pythium diseases

Leaf and head rot of Chinese cabbage (Brassica campestris pekinensis), caused by Pythium tracheiphilum, has led to harvest losses up to 50% since the disease appeared in Denmark in the 1980s. The disease differs from other Pythium–induced diseases since only adult plants are attacked. It appears that leaves are infected during contact with infested soil. Since no control measures were available, one potential fungicide and nine potentially antagonistic microorganisms, including four commercial BCA formulations, were tested for efficacy against leaf and head rot in the field. Agents were sprayed twice before head formation. Only treatments with IK726 controlled leaf and head rot. A 2-3 fold disease reduction was obtained with a spraying rate of 10⁹ conidia per m² in two years field trials conducted on naturally infested land. Further, the IK726 treatment increased percentage of marketable heads by 10% in both years. (Møller et al., 2003).

The efficacy of IK726 has also been tested against P. ultimum causing pre- and post emergence death of carrot. Seed coating and IK726-biopriming of carrot seed improves seedling establishment on growth media artificially infested with P. ultimum in growth chamber assays. In a field experiment, sowing of pathogen-free IK726-bioprimed seed significantly improved seedling emergence compared to pathogen-free primed seeds. This indicates that occurrence of IK726 on the seed protect against soil borne pathogens (Birgit Jensen, unpublished).

Ecological performance

The multi-target effect of C. rosea is probably related to its ability to thrive and survive under a wide range of temperature, nutrition and habitat conditions. IK726 reduces activity of pathogenic fungi causing problems on very different types of seeds, kept at high MC and temperatures from -1 to -15ºC, as shown above. Furthermore, IK726 significantly reduce seedling symptoms from cereal seed borne Fusarium culmorum at soil temperatures ranging from about 6 to 13ºC, and in growth chamber assays at 10, 15, 20 and 25ºC (Knudsen et al., 1995a; Jensen et al., 2000). Following seed application, IK726 reproduces and survives several months in the rhizosphere of barley and carrot from natural soils (Knudsen et al., 1995b; Johansen et al., submitted, Jensen et al., in prep).

The gfp-transformant IK726d11 resembling the wildtype strain IK726 in ecological fitness parameters was used for studies of fungal behaviour in different niches (Lübeck et al., 2002). Conidiogenesis, conidial germination and colonization were shown to take place in natural soil, in vermiculite, on carrot and barley seed, and on roots as well as on barley leaves. The fungal distribution on seed during carrot priming and Abies stratification was also studied, and a fungal affinity to the micropyle (carrot) and the testae (Abies) was observed (Jensen et al., 2004; Inge M.B. Knudsen, unpublished).

In summary, C. rosea IK726 has proved to be effective against seed and soil borne diseases in very different crops under quite diverse ecological conditions. However, the studies with biopriming, “bio”stratification and wet storage show that the isolate is favoured by humid conditions.

Acknowledgements

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References


Analysis of efficacy of a biocontrol agent to reduce the transmission of infection in damping-off epidemics

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Abstract: We demonstrate a simple analysis based on generic epidemiological processes to quantify the efficacy of Trichoderma harzianum to control damping-off disease caused by Rhizoctonia solani. The method quantifies the rate at which disease is transmitted in a population of plants, and we use the reduction in disease transmission as a measure for efficacy of the control agent. Using epidemics in replicated microcosms we show how the method can be used to quantify i) a reduction in the secondary transmission rate; ii) the effect of soil type on the efficacy of biocontrol and iii) the effect of the timing of the application on the efficacy of biocontrol.

Keywords: disease transmission, Rhizoctonia solani, Trianum, damping-off epidemic, soil type

Introduction

For many soil-borne pathogens, the reservoir of inoculum in soil is a critical component of the dynamics of an epidemic, with the source of inoculum being able to persist for long periods in the soil. Two processes therefore drive soil-borne epidemics: primary infection from resident (or incoming) inoculum, followed by newly infected host tissue from transmission between diseased and healthy plants (Gilligan, 2002). Widely-used empirical models for disease progress (such as the mono-molecular or logistic functions) cannot summarise these dynamics adequately. Nevertheless, these models are typically used to analyse the efficacy of control strategies on epidemics. Whilst this allows for an empirical comparison, it tells us little about how biocontrol reduces the transmission of infection from diseased to healthy plants in a population, nor does it discriminate between primary and secondary infection. Such a distinction is important as biocontrol can affect these components differentially (Kleczkowski et al., 1996). In this paper we present a simple analysis based on generic epidemiological processes to quantify the efficacy of the fungus Trichoderma harzianum strain T-22 to control damping-off disease caused by Rhizoctonia solani Kühn (AG 2-1, IMI385769). First, we demonstrate the method for replicated, controlled environment studies. Secondly, we show how the method can be used to compare the efficacy of biocontrol for different soil types. Finally, we show that we can also quantify the length of the period during which biocontrol was able to reduce secondary infection.

Materials and methods

Replicated microcosms to quantify dynamics of epidemics

Damping-off epidemics were studied in replicated microcosms comprising 160 radish plants (Raphanus sativus L., Cherry Belle), spaced 20 mm apart on a square lattice, grown in either a sterile sand (grade 16-30, Hepworth Minerals and Chemicals, Redhill, 10% wetness), a loam based soil (Westland Horticulture, wetness 30%), or peat (Levington, wetness 35%).
Epidemics were initiated by challenging 15 randomly selected plants with *R. solani*. Previously, we have shown that following the onset of disease, secondary spread occurs predominantly to nearest neighbouring plants. The biocontrol agent Trianum-P (Koppert), which contains the antagonistic fungus *Trichoderma harzianum* was added to provide an initial spore density of $4 \times 10^7$ conidia/g. For each treatment there were five replicated epidemics without biocontrol, and five in which Trianum-P was applied at the time of sowing. In addition Trianum-P was also applied 10 days earlier to the loam based soil to examine the effect of the timing of the application on the efficacy. In all microcosms, the time at which each plant became diseased was recorded for a period of 18 days to produce spatio-temporal maps of disease progress.

**Estimation of transmission rates**

We combine these spatio-temporal maps of disease progress with a generic *S*-I (susceptible-infected) model frequently used to describe plant epidemics, to obtain empirical estimates of the rate of disease transmission between infected and healthy plants (Otten et al., 2003; Otten et al., 2004). The basic idea behind the analysis is that epidemics are fuelled by (i) the number of contacts between infected (I) and susceptible (S) neighboring plants, and (ii) the probability of successful transmission of disease from an infected to a susceptible plant. The analysis is therefore based only on classes that can be observed, namely healthy and diseased. This enables a simple estimation of the rate of transmission of disease from an infected to a healthy neighbouring plant at each time interval during the epidemic. If the number of inoculations is known (as in our example) these rates can be calculated for primary as well as secondary infection. We propose that the efficacy of biocontrol can be measured as the reduction in the rate of transmission of disease.

**Results and discussion**

First we exemplify our analysis for replicated epidemics of damping-off disease caused by *R. solani* on radish seedlings grown in sterile sand. Inspection and analysis of the spatio-temporal maps of disease progress shows two sources of infection driving the epidemic, namely primary and secondary infection. Secondary infections dominated the epidemics via spread to neighbouring plants, with new infections each day at only a small percentage of the challenged plants (Figure 1a). The mean (n=5) temporal dynamics are summarised by disease progress curves (Figure 1b). There was less infection in the populations with biocontrol, though considerable variability was observed amongst replicated epidemics. Primary infection occurred mainly from day 5 till day 10, and was slightly reduced (13%) by biocontrol (9±0.5 and 7.8±0.9 plants per population, respectively). Most reduction (55%) occurred in plants that had become diseased through secondary infection (130±10 and 58±12 plants per population, respectively). Without further analysis, however, it is not possible to identify if this reduction results from the minor difference in primary infection which becomes amplified (Kleczykowski et al., 1996; Otten et al., 2003), or from a reduction in the secondary infection process.

**Soil type and efficacy of biocontrol**

We concentrate our analysis here on the efficacy of the control agent to reduce secondary infection, as primary infection was introduced artificially in our system, and secondary infection was the dominant driver of our epidemics. The rate at which disease is transmitted from an infected to a healthy plant increased initially with time but subsequently declined from day 11 onwards, as plant resistance increased (Figure 1c). Biocontrol significantly reduced the rate of disease transmission throughout the entire duration of the epidemic. We propose that a reduction in the rate of disease transmission is a good indicator of the efficacy of the biocontrol agent. For secondary infection, which dominated our epidemics, the efficacy
of biocontrol was approximately 37% in sand (Figure 1c, Table 1). Note that due to the non-linear nature of secondary infection a reduction of 37% in the transmission rate results in a 60% reduction in final levels of disease. In non-sterile environments, e.g. the loam based soil (which was found to suppress damping-off epidemics (Otten et al., 2004), and peat (which appeared to be conducive for damping-off epidemics compared to sand), the effect of the biocontrol agent on secondary infection was severely reduced (Table 1). The consequence of unsuccessful control of the secondary infection process is that once disease gets established into a population it is as likely to spread as in the untreated epidemic.

Figure 1. Effect of biocontrol agent *Trichoderma harzianum* on damping-off epidemics in sand caused by *R. solani*. (a, upper left) Typical example of the daily spread (black) of damping-off disease from previously infected plants (grey), which needs to be reduced by biocontrol. (b, upper right) The mean (black) and replicate (grey) cumulative disease progress curves for epidemics with (open) and without biocontrol (closed). (c bottom) Dynamics of secondary transmission rate with (open) and without (closed) biocontrol. The arrow indicates the reduction due to biocontrol.
Table 1. Mean efficacy (%) of biocontrol agent Trianum-P to reduce the rate of secondary transmission of disease in damping-off epidemics caused by *R. solani*, in various growth media

<table>
<thead>
<tr>
<th>Efficacy</th>
<th>Sand</th>
<th>Peat</th>
<th>Loam</th>
<th>Loam, applied early</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37±6.3</td>
<td>17±4.6</td>
<td>11±8.7</td>
<td>17.3±7.2</td>
</tr>
</tbody>
</table>

**Duration of efficacy of biocontrol**

Whereas for the epidemic in sand the efficacy was approximately at a constant level throughout the epidemic (Figure 1c), this is not always the case. For peat, the control was slightly more efficient during the middle phase of the epidemic; for loam there was a minor reduction in efficacy with time. We tested therefore if the efficacy could be enhanced by altering the time of application by applying it 10 days before sowing. Early application enhanced the efficacy of the control in loam based soil (Table 1). Possibly, the early application gave *Trichoderma* a better change to establish itself. However, our epidemiological analysis showed that a reduction was only achieved during the early stages of the epidemic (Figure 2). The control broke down rapidly and was virtually absent during the final stages.

![Figure 2](image-url)

Figure 2. Efficacy of biocontrol agent Trianum-P in a loam based soil applied 10 days before sowing in controlling damping-off epidemics caused by *R. solani*. Disease progress (a upper left) is significantly reduced, which is caused by a reduction in the rate of disease transmission during the first 12 days (b, upper right). (c bottom). Following successful control initially, the efficacy of the biocontrol agent declines rapidly during the epidemic.
Conclusions

Much research effort targets improving efficacy of biocontrol, yet surprisingly little research targets the development of new techniques to analyse their performance. We introduced an epidemiologically based measure for the efficacy of biocontrol. The method reveals how biocontrol affects the transmission of disease, which is one of the components driving epidemics. We demonstrated how such analyses can provide insight into the effect of soil type and timing of application on the efficacy of a control agent for damping-off epidemics. Application of this method can therefore be a crucial step in the development of biocontrol strategies as it identifies the extend at which a control agent is able to reduce transmission of disease and identifies when it fails.

Acknowledgement

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References

Recent developments in inoculum production and application, ecology and pathogenicity in the biocontrol agent *Coniothyrium minitans*

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Abstract: *Coniothyrium minitans* is a sclerotial mycoparasite of the plant pathogen *Sclerotinia sclerotiorum* and is used commercially as a disease biocontrol agent. However, knowledge of inoculum production and application for optimal efficacy is limited, ecological information is scarce, and little has been done to characterise the genes and gene products involved in this mycoparasitic interaction. Consequently, over the last few years, a collaborative series of studies examining these key features associated with biocontrol have been carried out. This paper briefly reviews the advances made in these areas and indicates future areas for study.

Key words: disease control, ecology, inoculum production, mycoparasite, pathogenicity, sclerotia

Introduction

*Coniothyrium minitans* has been known as a sclerotial mycoparasite and biocontrol agent (BCA) of the plant pathogen *Sclerotinia sclerotiorum* for many years (Whipps & Gerlagh, 1992) and two products Contans and KONI are available (Whipps & Davies, 2000). Nevertheless, the methods of inoculum production, ecology and factors controlling pathogenicity are still largely unexplored. Collaborative studies in the last few years have begun to address these areas of research and this paper briefly highlights some of the results obtained in these different areas and future research directions.

Inoculum production and application

A number of solid state fermentation processes for production of *C. minitans* have been explored (De Vrije et al., 2001). Recently, the ability of three *C. minitans* isolates (Conio, IVT1, and Contans) to infect sclerotia and decrease apothecial production of *S. sclerotiorum* was examined when they were applied as conidial suspensions (prepared from maize meal
perlite (MP) inoculum) and compared with C. *minitans* Conio applied as MP inoculum directly (Jones et al., 2003a). Application at $10^6$ to $10^7$ colony forming units cfu/cm consistently gave greater reduction of apothecial production than lower rates ($10^3$ to $10^4$ cfu/cm) and MP inocula were consistently more effective at reducing apothecial production than conidial suspensions. In general, there were few differences between the isolates of C. *minitans* applied as conidial suspensions. Experiments done at different times of the year indicated that temperature and soil moisture influenced apothecial production and mycoparasitism. Overall, these results indicate that inoculum rate and type, and appropriate timing are important factors in achieving reproducible reductions in apothecial production. However, the problem of cost effective production of inoculum for use at the high rates of application suggested for optimum activity of C. *minitans* needs to be addressed.

One approach is to use solid state fermentation in a packed bed reactor where yields in excess of $10^{14}$ conidia/m have been achieved for C. *minitans* growing on oats or hemp impregnated with glucose and yeast extract (Jones et al., 2004). However, the quality of the conidia produced and their subsequent survival needed to be assessed and a S. *sclerotiorum* sclerotia based infection test was devised for this purpose. Spray drying of conidia of C. *minitans* IVT1 maintained viability following storage for 6 months at 5°C, irrespective of whether the conidia were obtained from parts of the reactor exposed to high temperature (above 30°C), or from areas maintained at lower temperatures, more appropriate for mycelial growth. Without drying, conidial survival was poor. However, there was no correlation between conidial germination and ability of the conidial inoculum to infect sclerotia of S. *sclerotiorum* suggesting that conidial germination may not always reflect the quality of the inoculum of this biocontrol agent. C. *minitans* is clearly an effective sclerotial mycoparasite even if conidia are relatively old or have been stored poorly.

An alternative approach to solid state fermentation for conidial production is liquid fermentation. Both shaken and static liquid culture has been used to produce biomass of C. *minitans* with formation of pycnidia and conidia on the surface of the medium during static culture (McQuilken et al., 1997, 2002). However, only recently has conidial production in agitated liquid culture been studied (Cheng et al., 2003). Conidial production greater than $10^7$/ml was obtained with some but not all isolates examined, and was associated with formation of pycnidia on mycelial strands in potato dextrose broth. Conidia produced in liquid culture were equally effective at infecting sclerotia of S. *sclerotiorum* as those produced on potato dextrose agar. Conidia from liquid culture, formulated in kaolin powder survived for 6 months at 8°C with a loss of <1 log _10_ cfu/mg. Overall, these studies indicate that using appropriate nutrient sources and isolates, successful production of effective C. *minitans* conidia can be achieved in both solid state and liquid fermentation systems and that conidia produced in these systems can be formulated and stored satisfactorily.

**Ecology**

C. *minitans* has been shown to survive in soil for at least three years following application (McQuilken et al., 1995) but recently there has been interest in applying this BCA to occupy the vacant ecological niche that occurs following soil steaming (Bennett et al., 2003). When applied at $10^6$ cfu/g soil, C. *minitans* survived at that level in nonsterile, sterilised or pasteurised soil for 30 d. However, when applied at $10^3$ cfu/g soil in the same systems, little recovery was obtained from nonsterile soil, as other indigenous fungi grew on the medium used for enumeration. In pasteurised soil, when propagules of indigenous fungi had largely been destroyed, recovery was constant at the level of application. Significantly, when applied to sterilised soil, C. *minitans* eventually reached >$10^6$ cfu/g soil indicating that proliferation
and survival were likely to occur in steamed soil in a commercial situation. This needs to be explored further.

The lack of a selective medium for *C. minitans* has been a constant problem for ecological studies. However, the use of a hygromycin mutant strain (Jones 2003b) enabled detection of *C. minitans* in four soils and from decaying sclerotia after 8 weeks incubation at 20\(^\circ\)C. This was the first time that fungi colonising sclerotia already infected by *C. minitans* were shown to mask the detection of *C. minitans* from sclerotia rather than displacing the original mycoparasite.

**Pathogenicity factors**

Although there has been much research on the use of *C. minitans* as a biocontrol agent, little is known at the molecular level of the mechanisms by which it infects its host. At Warwick HRI, we are attempting to identify metabolites produced by *C. minitans* that may be involved in the sclerotial infection process and to identify, clone and characterise *C. minitans* genes required for pathogenicity against *S. sclerotiorum*.

At least three groups of metabolites, 3(2H)-benzofuranones, chromanes and the polyketide, macrosphelide A have been recovered from culture filtrates of *C. minitans* (Machida et al., 2001; McQuilken et al., 2003). Macrosphelide A was shown to be the major metabolite produced by *C. minitans* Conio and inhibited growth of *S. sclerotiorum* and *Sclerotium cepivorum* at concentrations of 46.6 and 2.9 µg/ml, respectively. The role of this and other metabolites from *C. minitans* in sclerotial infection is currently being studied at Warwick HRI (Tomprefa et al.). The spectrum of activity of this metabolite is also being examined.

In order to identify novel genes associated with sclerotial infection a series of knockout mutants were produced. This involved the development of transformation systems using both Restriction Enzyme Mediated DNA Integration (REMI) and *Agrobacterium tumefaciens* (Rogers et al., unpublished). Both techniques gave comparable numbers of transformants with 32 transformants/µg of DNA and 37 transformants/5x10\(^5\) germlings, respectively. Southern analysis has shown single-copy integration in 17% of REMI and 37% of *Agrobacterium* transformants. From a collection of over 3000 REMI-HindIII transformants and 1000 *Agrobacterium*-derived transformants, nine pathogenicity mutants, two auxotrophic mutants and 39 secondary metabolite mutants were identified. DNA flanking the sites of insertion in four REMI-derived pathogenicity mutants was recovered using plasmid rescue. Sequencing of one of these revealed two putative open reading frames (ORFs) with homology to (i) *Schizosaccharomyces pombe* RRM3/PIF1 helicase involved in mtDNA repair and recombination and (ii) a pisatin demethylase gene (PDA) from *Nectria haematococca*.

To recover wild-type copies of the putative pathogenicity genes, a *C. minitans* cosmid genomic library has been prepared and is currently being screened via macro-arrays. Insertional inactivation of pathogenicity genes is planned for the future.

**Acknowledgements**

We would like to thank the BBSRC, Defra, Horticulture LINK and the EU (project BIO4 CT98-0083 and Framework 6, project 001687, 2E-BCAs in crops) for financial support for this work.
References


TUSAL®, a commercial biocontrol formulation based on *Trichoderma*

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**Antonio Llobell², Enrique Monte³**

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**Abstract:** *Trichoderma* is a natural and cosmopolite fungus which offers an environmentally friendly approach to the management of plant diseases. It can be incorporated with cultural and physical control methods and integrated with limited chemical usage for effective IPM strategies. We have combined different strains of *Trichoderma* in both liquid (osmotically regulated) and solid (wettable powder or granules) formulations, for soil, plant and seed applications. The progress in formulation designs, production efficiencies and field results, is shown in crops such as sugar beet, lettuce, strawberry, pepper or tomato diseases. At present, one of the formulations based on *T. harzianum* and *T. viride* (TUSAL®) is being registered as fungicide in Spain and the EU.

**Key words:** biological control, *Trichoderma*, TUSAL®

**Introduction**

*Trichoderma* is a useful biocontrol agent of plant pathogens suitable for protecting or treating plants and plant material against infections and diseases caused by phytopathogenic organisms; to stimulate plant growth; to enhance root system; to induce systemic resistance in plants and to control biodeterioration agents of materials. We have combined different strains of *Trichoderma* in both liquid and solid formulations, not only for soil or plant applications but also for coated seeds. The progress in formulation designs, including production efficiencies and field results, is shown in pathosystems such as sugar beet damping-off, sugar beet Rhizoctonia root rot (Grondona et al., 2001b), rhizomania disease (Grondona et al., 2001a), lettuce drop (Campos et al., 2001), strawberry diseases (Monte et al., 2003) and pepper soil diseases. More than seventy trials were performed in different parts of Spain and other European countries with biocontrol formulations based on *Trichoderma*. One of the formulations based on *T. harzianum* and *T. viride* (TUSAL®) is being registered as fungicide in Spain and the EU.

**Materials and methods**

**Strains**

Single-spore cultures of *T. harzianum* NBT-T11 and *T. viride* NBT-T25 were maintained at –80°C on glycerol and cultured in the dark at 25°C on PDA until TUSAL® inoculum production. Both strains can be identified by ITS1/tef1 sequencing (Hermosa et al., 2004).

**Sugar beet trials**

Two sugar beet trials were carried out in two different locations of Castilla y León region (Spain): Vinaderos (Avila) and Baños de Cerrato (Palencia). Sugar beet seeds of two different
cultivars: Sheriff (SES/Advanta) and Granate (Lion Seeds) were coated with TUSAL®, and compared with chemical protected seeds (hymexazol 18 g/unit, and thiram 8 g/unit). Sheriff seeds were also pilled with TUSAL® and hymexazol (18 g/unit).

Greenhouse experiments were carried out in Olmedo (Valladolid) in trays with soil inoculated artificially with a R. solani strain (0.5% v/v), isolated from an infected sugar beet field in Ataquines (Valladolid). Three TUSAL® treatments were tested: 1) TUSAL®-coated sugar beet seeds were sown in the trays inoculated with R. solani (traditionally-sown sugar beet), 2) TUSAL®-coated sugar beet seeds were grown in paper-pots for six weeks before transplanting to the R. solani inoculated soil, 3) TUSAL® was applied to the paper-pot substrate before transplanting.

**Strawberry trials**

Three TUSAL® treatments were tested in hydroponic strawberry production trials in Almonte (Huelva, Spain). TUSAL® was applied by three ways: 1) colonizing the rockwool substrate before planting, 2) by strawberry plant immersion, and 3) through the irrigation system.

**Other trials**

More field trials were carried out in lettuce (Benicarló, Castellón), cotton (Lebrija, Sevilla) and asparagus (Huétor-Tájar, Granada), using TUSAL® to control soil-borne pathogens such as Sclerotinia sclerotiorum, Verticillium dahliae and Fusarium, respectively. Solid formulations of TUSAL® were used in these trials.

**Results and discussion**

Since it is difficult to summarize all the results obtained in our biocontrol trials during the last ten years, we are showing some examples of TUSAL® applications in different crops and pathosystems during the 2002 season.

**Sugar beet trials**

Vinaderos (Avila, Spain) field: Two different sugar beet seeds (Granate and Sheriff) coated with TUSAL® in a very hard R. solani-infected soil (Table 1) gave higher sugar yield than chemical fungicides: 3.7% for Granate and 15.7% for Sheriff.

**Table 1. Efficiency of TUSAL®-coated sugar beet seeds against R. solani. Vinaderos trial.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Roots (no./h)</th>
<th>Weight (Tm/h)</th>
<th>Polarization (%)</th>
<th>Sugar (kg/h)</th>
<th>Sugar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granate</td>
<td>70556</td>
<td>85.7</td>
<td>15.9</td>
<td>13806</td>
<td>100.0</td>
</tr>
<tr>
<td>Sheriff</td>
<td>68333</td>
<td>84.9</td>
<td>16.5</td>
<td>14315</td>
<td>103.7</td>
</tr>
<tr>
<td>Sheriff + hymexazol</td>
<td>97222</td>
<td>112.9</td>
<td>16.6</td>
<td>18673</td>
<td>104.2</td>
</tr>
</tbody>
</table>

**Table 2. Efficiency of TUSAL®-coated sugar beet seeds vs R. solani. Baños de Cerrato trial.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Roots (no./h)</th>
<th>Weight (Tm/ha)</th>
<th>Polarization (%)</th>
<th>Sugar (kg/ha)</th>
<th>Sugar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheriff</td>
<td>82222</td>
<td>106.4</td>
<td>16.8</td>
<td>17917</td>
<td>100.0</td>
</tr>
<tr>
<td>Sheriff TUSAL</td>
<td>103333</td>
<td>117.8</td>
<td>16.4</td>
<td>19295</td>
<td>107.7</td>
</tr>
<tr>
<td>Sheriff TUSAL + hymexazol</td>
<td>97222</td>
<td>112.9</td>
<td>16.6</td>
<td>18673</td>
<td>104.2</td>
</tr>
</tbody>
</table>
Figure 1. TUSAL treatments against *Rhizoctonia solani* in traditional-sowed and transplanted sugar beets. Greenhouse trial. Olmedo (Valladolid, Spain). 2002

Figure 2. Hydroponic strawberry (fruit g/plot). Almonte (Huelva, Spain). Campaign 2002-03.

Baños de Cerrato (Palencia, Spain) field: Sheriff seeds protected with hymexazol + thiram (standard), TUSAL®, and TUSAL® + hymexazol were sowed in a *R. solani* medium-infected soil (Table 2). The maximum sugar yield was obtained with TUSAL® (107.7%), followed by TUSAL® + hymexazol (104.2%) and standard (100%).
Olmedo (Valladolid, Spain) greenhouse: Sheriff seeds were tested in soils infected with *R. solani* at greenhouse level (Figure 1). The best control of *R. solani* was detected in paper-pots sowed with TUSAL®-protected seeds, and paper pots with TUSAL® added to the substrate. Good control was also observed in the seeds treated with TUSAL® and sowed by traditional way.

**Strawberry trials**
Almonte (Huelva, Spain) soil-less fruit production: a feasible alternative to soil fumigation is to produce strawberries in soil-less systems In this trial, rockwool substrates colonized by TUSAL® plus plant immersion in TUSAL® conidial suspensions gave the maximum yield of fruits (Figure 2).

**Other trials**
Encouraging results were obtained in lettuce where the percentage of dead plants decreased 40-45% after TUSAL® applications. TUSAL® increased 18% the weight of fiber in cotton trials. TUSAL® applied to the soil decreased 50% the incidence of *Fusarium* in asparagus.

**References**
Control of foliar and wood diseases
Application of a yeast, *Pichia anomala* strain WRL-076 to control *Aspergillus flavus* for reducing aflatoxin in pistachio and almond

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Abstract: Aflatoxin contamination is associated with wounding in pistachio nuts. The efficacy of *Pichia anomala* was assessed by artificially wounding pistachio nuts in the orchard. Production of *Aspergillus flavus* spores in wounded nuts sprayed with yeast was reduced by 77-99% compared to control. Furthermore colonization of *A. flavus* on wounded pistachio nuts was prevented by the presence of yeast on wounded nuts by 3-5 fold.

Key words: yeast, *Aspergillus flavus*, aflatoxin, pistachio, almond, biocontrol, wounding, colonization, spore production

Introduction

The Food and Agriculture Organization (FAO) estimates that 25% of the world’s food crops are affected by mycotoxins, of which the most notorious are aflatoxins. Contamination of aflatoxin in tree nuts, peanuts, corn and cottonseed has been recognized as a serious food safety hazard to both human and animal. Aflatoxin levels of 2-4 ppb have been declared mandatory by importing European Countries (Ellis et al., 1991; Commission of the European Community, 1998). Even very low levels of infection of the nuts by *A. flavus* can result in aflatoxin levels above these mandatory standards. Managing aflatoxin contamination via biological control using yeast as an antagonist is a promising approach (Hua et al., 1998; Janisiewicz & Korsten, 2002).

A bioassay has been developed to screen for effective yeast inhibiting both the growth of the *Aspergillus flavus* and aflatoxin production (Hua et al., 1999). One particular yeast, *Pichia anomala* strain WRL-076 was tested further for its antagonistic activities to reduce spore production of toxigenic and atoxigenic isolates of *A. flavus* in pistachio flowers and nut-fruits as well as in almond and pistachio leaves. Both sterilized and non-sterilized plant materials were tested in lab experiments. Spore production of *A. flavus* was reduced by 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; Hua, 2000; Hua, 2002; Hua, 2003). In this report, we describe a procedure to assess the efficacy of biocontrol yeast in pistachio orchard.

Material and methods

Yeast culture

The yeast strain, *P. anomala* WRL-076 was maintained on potato-dextrose agar (PDA, Difco, Detroit, MI). Yeast suspension was prepared in sterile water. A Beckman-Coulter Multisizer was used to determine the number of yeast cells. Microorganisms were eluted in Tween 80 solution from the experimental samples by shaking and sonicating in flasks. A spiral plating system was used to spread the microorganisms onto nutrient agar. Viable counts of yeast were
determined by spreading the yeast on dichloran rose bengal chloramphenicol (DRBC) agar plate.

**Field evaluation of the efficacy of the biocontrol agent, P. anomala WRL-076**

Pistachio nut-fruits on the tree were individually wounded with a dental needle and sprayed with aqueous suspension of yeasts at $3 \times 10^7$ cells/ml on 10/07/03 (Experiment 1) and 08/08/03 (Experiment 2) respectively. The wounded nut-fruits without yeast-spray were used as controls. Wounded nut-fruit was hand picked from the tree and immediately placed to a special agar medium on 08/08/03 (Experiment 1) and 15/09/03 (Experiment 2). The nut-fruits were incubated at $28^\circ C$ for eight days. Colonization of yeast and *A. flavus* on wounded nut-fruits were scored. The viable fungal and yeast counts on individual nut were enumerated by standard microbiological techniques.

**Results and discussion**

**Effect of yeast on colonization of wounded pistachio**

In 2003, two experiments were conducted in a pistachio orchard in Winters, California. Pistachio nut-fruits on the tree were wounded and sprayed with yeast and collected for analysis. In order to see the variation among the nuts, every single nut collected was analysed for colonization of *A. flavus* and viable spores. In experiment 1, colonization of nut fruits by *A. flavus* was 6.8% for the control and 2.5% for wounded nut-fruits treated with yeast. In experiment 2, the percent colonization of *A. flavus* on nut-fruits was 27.1% for the control and 5.1% for the yeast treated nut-fruits respectively. Higher colonization of *A. flavus* in experiment 1 than in experiment 2 indicted that the population of *A. flavus* in the orchard was higher in August than in July. The results are summarized in Figures 1 and 2. The results demonstrate that the yeast, *P. anomala*, significantly reduced the frequency of colonization by *A. flavus* in wounded pistachios by three to five fold.

![Figure 1. Effect of yeast on colonization of A. flavus on wounded Pistachio. Nuts were wounded on 10/07/03 and harvested on 08/08/03 for analysis.](image)

**Inhibition of spore production of A. flavus by yeast**

The CFU of *A. flavus* spores from each single nut was enumerated. The distribution of spores produced in all wounded nuts infected by *A. flavus* is summarized in Figures 3 and 4. In experiment 1, the average spore production for the control and yeast sprayed nuts was $1.4 \times 10^7$ and $3.1 \times 10^3$ respectively. Average spore production in *A. flavus* infected nuts was $5.6 \times 10^6$ and
1.3x10^6 respectively for the control and yeast sprayed nuts in experiment 2. The experiments demonstrated that the yeast, *P. anomala* can modulate spore production of *A. flavus* in wounded the pistachio nut-fruits. A reduction of spore number was observed in the range of 77% and 99% for experiment 1 and experiment 2, respectively.

Figure 2. Effect of yeast on colonization of *A. flavus* on wounded Pistachio. Nuts were wounded on 08/08/03 and collected on 15/09/03 for analysis.

Figure 3. Spore production of *A. flavus*. Nuts were wounded on 10/07/03 and collected on 08/08/03 for enumeration of *A. flavus* spores.

Figure 4. Spore production of *A. flavus*. Nuts were wounded on 08/08/03 and collected on 15/09/03 for enumeration of *A. flavus* spores.
**Rationale for using wounded pistachio nut-fruits**

Aflatoxin contamination is associated with wounding in corn, peanut, cotton seed and tree nut (Diener et al., 1987; Payne, 1992; Cotty et al., 1994; Hua et al., 1998). Assessment of the efficacy of *P. anomala* has been achieved by mechanically wounding pistachio nuts on the tree in the orchard to increase the number of wounded nuts. The results clearly demonstrate that the production of *A. flavus* spores was drastically inhibited by spraying yeast onto wounded pistachio nuts. One can anticipate that field spraying of this effective yeast to pistachio trees will decrease the population of *A. flavus* in the orchards. The outcome will be a reduction of aflatoxin contamination in the edible nuts. Using pistachio as a model system, similar results can be predicted for almond.

**Acknowledgements**

We thank S. B. Ly, S. Kwang, L. T. Fang and T. Chen for technical assistance; D. E. Parfitt for discussion.

**References**


**Pseudomonas fluorescens EPS62e, a potential biological control agent of fire blight**

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**Abstract:** A biological control agent, *Pseudomonas fluorescens* EPS62e, was isolated from the surface of a pear fruit using a selective enrichment procedure. The strain was selected among 600 isolates of *P. fluorescens* and *Pantoea agglomerans* obtained from flowers, fruits and leaves of rosaceous plants in a survey performed through several geographic areas of Spain. Strain EPS62e was evaluated for suppression of immature fruit, blossom and shoot infections, under controlled environment conditions, providing control levels similar to control with copper or antibiotic compounds. The strain also colonized and survived well in flowers and wounds on immature fruit. Pre-emptive exclusion of the pathogen *E. amylovora* by surface colonization and nutrients depletion, and cell-to-cell interaction appear to be the main mechanisms of biocontrol. Specific methods of analysis of EPS62e were developed using strain-specific molecular markers based on sequence characterized amplified regions (SCAR). Two couples of SCAR primers gave unique and specific amplification products for EPS62e, whereas they gave no amplification with 162 strains of *P. fluorescens* and 71 strains of other closely related species.

**Keywords:** Fire blight, biocontrol, mechanisms, SCAR

**Introduction**

Fire blight, caused by *Erwinia amylovora*, is a serious disease affecting several rosaceous plants with great commercial and economic interest. Disease may be controlled commercially by the application of chemicals (copper compounds, antibiotics). Many chemical agents have low efficacy or cause phytotoxicity, and streptomycin, the most effective antibiotic, is not approved for use in many countries. Besides, where streptomycin is used, the pathogen may develop resistance (Thomson et al., 1993; Stockwell et al., 1996). Therefore, in the absence of suitable chemical control agents, biological control could provide a useful alternative or complement of other measures. This may be achieved using various naturally occurring microbial antagonists, including the phylloplane inhabitants *Erwinia herbicola* and *Pseudomonas fluorescens* (Montesinos et al., 1996). These species have been used for biological control of fire blight (Wilson & Lindow, 1993; Kearns & Hale, 1996). The aim of this study was to select a biocontrol agent of fire blight, determine the potential mechanism of biocontrol and develop a specific DNA marker for the traceability of the strain.

**Materials and methods**

**Selection the biocontrol agent**
Screening was performed using different bioassays. Fruit assays were performed in whole immature pear fruits of Passe Crassane cultivar that were wounded. Blossom assay was performed in individual pear flowers of Doyenne du Comice cultivar that were maintained...
with the cut peduncle submerged in a sucrose solution. Finally, self-rooted pear plants of cultivar Conference with wounded leaves were used in a young shoot assay. Two trials were performed in fruits, testing 533 isolates in the first assay and the 61 best antagonists in the second. The seven best antagonists in fruit assay were tested in the blossom assay and finally strain EPS62e was tested at two doses in the shoot assay. Experimental design consisted of three repetitions per treatment with three fruits per repetition in immature pear fruit assay, eight flowers per repetition in blossom assay and three plants per repetition in young shoots assay. A non treated control was included in each assay. Disease incidence was evaluated after nine to ten days of pathogen inoculation. ANOVA was performed to test the effect of putative biocontrol agents in the reduction of disease incidence. The means were separated according to the Tukey’s test at \( P \leq 0.05 \).

**Colonization studies**

The ability of EPS62e to survive, colonize and inhibit growth of *E. amylovora* EPS101 in immature pear wounds and hypanthium of pear flowers when both bacteria were coinoculated was investigated. Passe Crassane wounded fruits and Doyenne du Comice flowers obtained from branches forced to bloom were used. Fruit wounds and hypanthium of flowers were inoculated with a spontaneous mutant of EPS62e resistant to nalidixic acid before of inoculation with a spontaneous mutant of EPS101 to rifampicin. Two controls were included, one inoculated only with EPS62e, and another inoculated only with EPS101. Three repetitions of three flowers or fruits per repetition were used for each treatment. Population levels of EPS62e and EPS101 were determined at 0, 4, 15, 24, 48 and 72 or 96 h.

**Interaction studies**

The study of direct interaction was performed *in vitro* using the method described by Janisiewicz et al. (2000) and used for studies of determination of mechanisms of action (Bonaterra et al., 2003). The test was done in tissue culture plates with 24 wells per plate containing Milli-cell culture plate inserts as the inner compartment. The culture medium was immature pear fruit juice. Treatments consisted of different EPS62e/EPS101 ratios, the existence or not of direct contact between cell strains and different pear juice concentration (1% or 10%). EPS62e was inoculated at \( 10^9, 10^8 \) and \( 10^7 \) cfu/ml while EPS101 was inoculated at \( 10^7 \) cfu/ml. In treatments without direct contact, the strains were separated through a semi-permeable membrane, while in treatments with direct contact the strains were cultured in the same compartment. A non-treated control inoculated with water instead of EPS62e was included. Each treatment was repeated three times. Population levels of *E. amylovora* were determined after 48 h of incubation at 25°C.

**Development of a strain-specific PCR-marker**

In order to develop Sequence Characterized Amplified Region (SCAR) markers for EPS62e a RAPD technique was used. Between several fragments amplified in the EPS62e RAPD profile, three fragments were selected which differentiated our strain from other *P. fluorescens*. These fragments were subsequently cloned and fully sequenced. The sequences were compared with the GenBank database to define the regions which had no homology with known DNA sequences. These regions where entered into the Primer Express™ software (Applied PE Biosystems), and a set of SCAR primers was designed for the three sequences. These three pairs of SCAR primers, 450f-450r, 900f-900r and 1000f-1000r where evaluated for their specificity in front of 162 *P. fluorescens* strains and other 71 related species.
Results

Selection of the biocontrol agent
In the first trial performed in immature fruit assay, it was observed that most of the isolates had certain efficacy in the inhibition of infections compared to the non-treated control, but only around 10% of the screened isolates were very effective (>90%). Also, only seven out of 61 antagonists retested for its efficacy in immature fruits maintained a high efficacy (>50%) in the inhibition of infections. In the blossom assay, the seven best antagonists previously selected in immature fruit assay were tested. Only strains EPS283e, EPS684, EPS734 and EPS62e showed significant control of blossom blight after 8 days of pathogen inoculation ($P \leq 0.001$). Strain EPS62e was the most effective with an efficacy of 83% in the reduction of the incidence of infections. Finally, in the shoot assay, it was observed that treatments with EPS62e reduced significantly the incidence of *E. amylovora* infections. Besides, significant differences between the treatments were observed. The efficacy was around 96% when EPS62e was applied at $10^8$ cfu/mL, and around 70% when was applied at $10^7$ cfu/mL.

Colonization studies
EPS62e colonized well the fruit wounds and flowers’ surface acquiring stable populations levels around $1.5 \times 10^7$ cfu/wound or cfu/flower. Besides, the growth of EPS62e was not influenced by the presence of *E. amylovora*. In contrast, the growth of *E. amylovora* especially in fruit wounds and flowers’ surface was inhibited by EPS62e. Thus, in fruit wounds, the populations levels of *E. amylovora* were reduced from $1.6 \times 10^9$ cfu/wound, when was inoculated alone to $4.3 \times 10^3$ cfu/wound, when was co-inoculated with EPS62e. While, in flowers, the populations levels were reduced from $1.9 \times 10^8$ cfu/flower to $1.7 \times 10^6$ cfu/flower.

Interaction studies
Growth of *E. amylovora* EPS101 was inhibited when cells of EPS62e were added to pear juice medium. When the strains were cultured separately through a semi-permeable membrane in 10% pear juice, growth of EPS101 was not significantly affected at 1:1 and 10:1 EPS62e/EPS101 ratios, but was reduced significantly at a ratio of 100:1 ($F=346.17$, $P \leq 0.0001$). In contrast, when the strains were co-cultured, all the treatments had a significant effect in the reduction of growth of EPS101 in comparison to the non-treated control (without EPS62e). In addition, the level of inhibition of EPS101 increased with the ratio, with highest values at 100:1. When bacteria were cultured in diluted pear juice (1%), significant reduction of growth of EPS101 was observed in all treatments in comparison to the non-treated control, including when EPS62e and EPS101 were cultured separately ($F=496.06$, $P \leq 0.0001$) and the inhibition of EPS101 increased with the increase of the EPS62e/EPS101 ratio.

Development of a strain-specific PCR-marker
The three EPS62e RAPD fragments selected and sequenced showed low levels of homology against the known sequences from the GenBank database. These results allowed the design of three SCAR primer pairs, that were tested for specificity against 162 *P. fluorescens* and 71 strains of other closely related species. Two SCAR primer sets, 450f-450r and 900f-900r, were specific for the amplification of EPS62e DNA, and gave no signal with the rest of the strains tested. However, the SCAR primer pair 1000f-1000r was semi-specific, because it amplified the same sequence in nine other *P. fluorescens* strains.

Discussion
EPS62e showed the ability to control infections produced by *E. amylovora* in immature fruits, flowers and shoots under controlled environment conditions, providing control levels similar to chemical control with copper or antibiotic compounds. EPS62e also showed the ability to colonize
and survive well in wounds of immature apple and pear fruits and in the pear flower surface under controlled environment conditions, showing also the ability to inhibit the growth of *E. amylovora* in these plant organs. Interaction studies have confirmed that direct interaction is needed for the inhibition of *E. amylovora* and nutrient competition can also be involved in the control process. This is probably occurs in immature pear fruits, flowers and whole plants, though other mechanisms may be involved like competitive exclusion by pre-emptive colonization. Therefore, the pre-emptive exclusion of *E. amylovora* by surface colonization, nutrients depletion, and cell-to-cell antagonistic interaction is the main cause of fire blight suppression by EPS62e. Finally, two couples of SCAR primers were developed and gave unique and specific amplification products for EPS62e. These molecular markers offer a simple and unambiguously detection system for the biocontrol agent EPS62e suitable for monitoring and colonization studies.

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Biocontrol agents against downy mildew of Grape: an ultrastructural study

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Plasmopara viticola (B. et C.) Berl. et De Toni, the grapevine downy mildew pathogen, causes an economically very important disease. It is particularly destructive in viticultural regions with warm, wet conditions during the growing season, including parts of Europe and North America. The aim of this work was to find and test biocontrol agents (BCAs) against P. viticola to limit the use of chemicals in organic agriculture. Several microorganisms were sampled from grapevine leaves in plants situated in an abandoned vineyard individuated in Tuscany (Central Italy). The microorganisms were isolated culturing in PDA medium small leaf sections-to select only endophytes-, and maintaining in thermostat at 25°C. 126 isolates were then purified and finally tested against grape downy mildew according to the screening system described by Pertot et al., 2003. Among the 126 isolates, five fungal microorganisms inhibited the sporulation of P. viticola in vitro. They were identified as all belonging to Alternaria alternata species. An ultrastructural analysis was carried out to know the cellular interactions between the pathogen and Alternaria alternata in the grape leaf tissue. A test to demonstrate the main activity of Alternaria alternata against P. viticola was also performed.
Effect of relative humidity on the efficacy of mycoparasitic fungi and antagonistic bacteria towards cucurbit powdery mildew

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Abstract: Podosphaera fusca is the causal agent of cucurbit powdery mildew in Southern Spain. The most promising biological methods for its control involve the use of mycoparasitic fungi and antibiotic-producing bacteria, however, their biological control performance are strongly dependent on environmental conditions. In this work we show the abilities of two commercial mycoparasites, Ampelomyces quisqualis and Lecanicillium lecanii, and four Bacillus spp. strains isolated in our laboratory, to manage powdery mildew disease on melon seedlings maintained under different regimes of relative humidity, suggesting the possibility of a similar performance under greenhouse conditions.

Key words: Ampelomyces quisqualis, Bacillus, cucurbits, Lecanicillium lecanii, Podosphaera fusca

Introduction

Powdery mildews are some of the most important diseases of cucurbits world-wide. The disease induces as the most characteristic visual symptom a whitish, talcum-like, powdery growth, developing on both leaf surfaces, petioles and stems; the reduction of fruit quality and crop yield being the most striking aspect of disease loss (Zitter et al., 1996). On cucurbits the disease can be caused by two fungal species, Golovinomyces cichoracearum or Podosphaera fusca, in Southern Spain, however, P. fusca has been identified as the sole cause of the disease (Del Pino et al., 2002). The main means of disease control are the use of resistant cultivars and repeated applications of fungicides. Nevertheless, resistant cultivars are not available for all cucurbit crops and it has been recently reported increasing frequencies of resistance to certain fungicides such as strobilurins (Fernández-Ortuño et al., 2004). All these constraints, together with the current public attitude and environmental concerns towards the use of chemicals, have led to the search of alternative methods and favoured biological approaches to control powdery mildews (Kiss, 2003).

In the case of powdery mildew, relative humidity has been by far the most important limiting factor for the efficacy of biological control agents (Verhaar et al., 1999). Since powdery mildews have mostly an ectotrophic life cycle, one can assume that they can be easy targets for hyperparasites and antibiotic-producing microorganisms (Bélanger et al., 1998). In previous works we have reported the ability of two commercial mycoparasitic fungi, Ampelomyces quisqualis and Lecanicillium lecanii, and four bacterial isolates identified as Bacillus spp. to control powdery mildew disease by an in vitro test using melon detached leaves (Romero et al., 2003, 2004). The aim of this work was to evaluate the efficacy of those biological control agents under different regimes of relative humidity.
Material and methods

Microorganisms and culture conditions
The isolate SF26 of *P. fusca* race 1 was routinely grown on cotyledons of zucchini cv. Negro Belleza, maintained *in vitro* as described by Álvarez & Torés (1997). The mycoparasitic fungi *A. quisqualis* and *L. lecanii* were used as the commercial formulations AQ10® (Ecogen) and Mycotal® (Koppert Biological Systems), respectively. Bacterial strains were routinely grown in nutrient agar plates at 28°C for 24 h.

Biological control test
Plants of the susceptible melon cv. Rochet at stage of third leaf completely expanded were used. A suspension of *P. fusca* (10⁵ conidia/ml) was spread onto the second and third leaves. Spore suspensions of mycoparasites were adjusted to 5·10⁶/ml and applied 3 days after *P. fusca* inoculation. Fresh bacterial liquid cultures were grown from frozen stocks in nutrient broth. Inocula were composed by bacterial cells harvested in stationary-phase of growth (48 h) and were applied 4 hours before pathogen challenge. Inoculated seedlings were maintained at 25°C under a 16h photoperiod, 3800 lux intensity and different regimes of relative humidity (RH).

Disease severity and conidia counts
Disease severity was estimated by percentage quantification of the leaf area covered with powdery mildew as previously described (Romero et al., 2003). The degree of powdery mildew conidiation was evaluated by conidia counts as reported earlier (Romero et al., 2004).

Results and discussion
To confirm the control abilities of the fungal and bacterial agents observed in detached leaf assays, biological control tests were carried out using melon seedlings maintained in growth chambers. As shown by the percentage of leaf area covered by powdery mildew (Table 1) the mycoparasitic fungi were more efficient at 90-95% RH, achieving disease reduction values of 85-92%, whereas below 80% RH disease reduction dropped to 63%. Interestingly, when mycoparasites were applied together, biocontrol results improved substantially with respect to each one alone, at least under sub-optimum conditions of RH, suggesting a sort of complementary effect that should be further investigated. Unfortunately we do not have the data corresponding to 90-95% RH, although it seems to be difficult to improve biocontrol more than 95% disease reduction.

Like other foliar phytopathogenic fungi, *P. fusca* profusely produces asexual conidia, thereby it is important to limit conidial production in order to successfully control the disease. To gain an insight into the potential effect of biocontrol agents on powdery mildew spreading, *P. fusca* conidiation degree was also evaluated. As it can be seen in Table 1, RH had an impact on the effect of mycoparasites on conidiation, similar to the effect on disease. Below 80% RH the mycoparasites were able to reduce the number of conidia by 60-65%, whereas, at 90-95% RH the reduction of conidiation was up to 93%. In addition, results similar to those observed for disease symptoms (leaf surface covered by powdery mildew) were obtained when the mycoparasites were applied together. A synergistic effect was observed, achieving conidiation reductions of 77-93 % under sub-optimum conditions of RH.

Similar experiments were carried out with four *Bacillus* spp. strains (Table 2). All strains managed to reduce disease symptoms at either 75 or 90% RH, although the best biocontrol results were obtained at 90-95% RH, achieving levels of disease reduction ranging from 60-80%. At 75-80% RH, however, with a better infection degree according to the untreated
controls (67% of leaf surface covered by powdery mildew), the percentages of disease reduction were lower (44-60%). Like in the case of mycoparasites, bacterial agents were also able to reduce conidiation of *P. fusca*, 90-95% RH being again the optimum condition to achieve reduction of conidiation (80-94%).

Table 1. Severity of powdery mildew on melon seedlings treated with mycoparasitic fungi under different regimes of relative humidity. Infection degree was assessed as percentage of leaf area covered by powdery mildew 16 days after application of mycoparasites. Conidiation degree of *P. fusca* is also indicated. The corresponding percentages of disease and conidiation reductions achieved by mycoparasites are given in brackets.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>75-80% RH</th>
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<th>80-85% RH</th>
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<th>90-95% RH</th>
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<tr>
<td></td>
<td>Leaf area</td>
<td>Conidiation</td>
<td>Leaf area</td>
<td>Conidiation</td>
<td>Leaf area</td>
<td>Conidiation</td>
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<tr>
<td></td>
<td>cover (%)</td>
<td>(conidia/cm²)</td>
<td>cover (%)</td>
<td>(conidia/cm²)</td>
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<td>(conidia/cm²)</td>
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<tr>
<td>Untreated</td>
<td>47</td>
<td>5.5·10⁴</td>
<td>66</td>
<td>1.4·10⁵</td>
<td>80</td>
<td>1.4·10⁷</td>
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<tr>
<td>Water</td>
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<td>4.8·10⁵ (13)</td>
<td>63 (5)</td>
<td>1.4·10⁵ (0)</td>
<td>68 (14)</td>
<td>1.3·10⁵ (10)</td>
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<tr>
<td><em>A. quisqualis</em></td>
<td>18 (63)</td>
<td>1.9·10⁵ (65)</td>
<td>27 (63)</td>
<td>3.7·10⁴ (77)</td>
<td>6 (92)</td>
<td>9.4·10⁷ (93)</td>
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<tr>
<td><em>L. lecanii</em></td>
<td>18 (63)</td>
<td>2.2·10⁵ (60)</td>
<td>23 (65)</td>
<td>5.0·10⁴ (67)</td>
<td>12 (85)</td>
<td>1.5·10⁷ (89)</td>
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<tr>
<td><em>Aq + Ll</em></td>
<td>14 (77)</td>
<td>1.5·10⁴ (77)</td>
<td>7 (89)</td>
<td>8.5·10³ (93)</td>
<td>nd³</td>
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</table>

ᵃ Values expressed as percentage of leaf area covered by powdery mildew or *P. fusca* conidia cm⁻² referred to untreated controls.
ᵇ Combined treatment with *A. quisqualis* and *L. lecanii*.
ᶜ Not determined.

Table 2. Severity of powdery mildew on melon seedlings treated with antagonistic bacteria under different regimes of relative humidity. Infection degree was assessed as percentage of leaf area covered by powdery mildew 16 days after application of *Bacillus* spp. strains. Sporulation degree of *P. fusca* is also indicated. The corresponding percentages of disease and sporulation reductions achieved by bacterial agents are given in brackets.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>75-80% RH</th>
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<th>90-95% RH</th>
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<td>Leaf area</td>
<td>Conidiation</td>
<td>Leaf area</td>
<td>Conidiation</td>
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<tr>
<td></td>
<td>cover (%)</td>
<td>(conidia/cm²)</td>
<td>cover (%)</td>
<td>(conidia/cm²)</td>
</tr>
<tr>
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<td>67</td>
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<td>46</td>
<td>9.9·10⁴</td>
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<tr>
<td>UMAF6614</td>
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<td>4.3·10⁴ (68)</td>
<td>10 (78)</td>
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</table>

ᵃ Values expressed as percentage of leaf area covered by powdery mildew or *P. fusca* conidia/cm² referred to untreated controls.

The fungal and bacterial biocontrol agents used in this study have proved to reduce the severity of powdery mildew caused by *P. fusca* on melon seedlings, limiting both development and growth of hyphae and production of conidia. However, it has been also clearly evidenced that relative humidity is a key factor for their efficacy, in such a way that to
achieve an effective control of the disease, values of RH above 85% are needed. This requirement makes more suitable the use of these biological control agents in greenhouse systems, wherein it can be possible to maintain the relative humidity at those levels. In the light of these data, it is necessary to carry out further research on the biocontrol effectiveness of these microorganisms under different environmental milieus, such as field and greenhouse conditions, prior to developing integrated control strategies including these fungi and bacteria as biological control agents, to minimize the dependence on chemicals of cucurbit powdery mildew management.

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References


Latest results on the biocontrol of fire blight in Germany

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Abstract: As alternatives to the antibiotic streptomycin against fire blight bacterial antagonists and a natural product were tested on their efficacy in greenhouse and field experiments. The antagonistic strain of *Rhanella aquatilis* - Ra39 in combination with aromatic compounds could increase the efficacy up to 68%. The etheric oil compound BioZell-2000B was able to reduce blossom infection up to 52% and is proposed as a bioproduct for fire blight in ecological farming.

Key words: fire blight, biocontrol, bacterial antagonists, natural product

Introduction

Research on alternatives to the antibiotic streptomycin for the control of fire blight in pome fruits has become of main interest in German and European fruit growing, as permission for the use of antibiotics in EEC-countries can be withdrawn in future time. Thus the development of biologicals on the basis of antagonism and natural products against the pathogen have been undertaken in germany especially by the Federal Research Centre (BBA) in cooperation with plant protection service. Latest results with antagonistic bacteria and a natural product based on an etheric oil of thyme, BioZell-2000B, which was developed together with Turkish colleagues (Yegen et al., 2002) will be presented.

Results and discussion

Studies on bacterial antagonists

First screening of several potential antagonists of more than 120 isolates, showed inhibitory effects mainly of the species *Pseudomonas fluorescens*, *Pantoea agglomerans* and *Bacillus subtilis* (Zeller & Wolf, 1996), later on also from the epiphytic bacterium *Rahnella aquatilis* (Laux et al., 2002). Moreover in field experiments efficacies up to 61% could be observed (Table 1) but without a similar control effect to streptomycin. In order to increase the efficacy of strain Ra39, the antagonist was combined with aromatic compounds, as it was lower sensitive to benzoate as the fire blight pathogen in vitro and was able to use this bactericidal substances as nutritive source (Figure 1) In field experiments the combination of Ra39 and Na-Benzoate was nearly comparable in the efficacy to streptomycin, 68 to 77%. Also another combination with the growth regulator REGALIS (APOGEE) showed high control effect of 68% (Figure 2).

Studies on natural products

As another biological alternative the plant strenghtener BioZell-2000B, a compound out of 70% Thyme oil from *Thymbra spicata*, which has been registered in Turkey as a plant protection substance (Yegen et al., 2002), was tested on its efficacy against fire blight under greenhouse and field conditions.
Table 1. Efficacy of bacterial antagonists against blossom infection, 1998-2001

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Origin</th>
<th>Efficacy (%)</th>
</tr>
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<tbody>
<tr>
<td><em>Bacillus subtilis</em> BsBD 170 (BIOPRO)</td>
<td>Soil</td>
<td>30-60</td>
</tr>
<tr>
<td><em>Rahnella aquatilis</em> Ra39</td>
<td>Apple blossom (Steinbrenner, 1991)</td>
<td>39-53</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td>Shoot tissue</td>
<td>45-58</td>
</tr>
<tr>
<td><em>Pantoea agglomerans</em> Pa21889</td>
<td>Apple Blossom (Steinbrenner, 1991)</td>
<td>50-61</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces griseus</em></td>
<td></td>
<td>68-80</td>
</tr>
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</table>

Figure 1. Effect of Na-benzoate on the growth of Ra39 and *E. amylovora* strain-Ea 7/74 *in vitro*

Figure 2. Fire blight control with Ra 39 in different combination on Golden Delicious after natural infection, 2003

Firstly, under *in vitro* conditions in a concentration of 0.05% no direct effect of BioZell-2000B on the pathogen could be observed indicating the plant strengthener effect of the compound. In greenhouse symptom development on leaves of the highly susceptible apple rootstock was markedly reduced after two days induction time with BioZell-2000B (Figure
3). Under field conditions on apple variety Boskoop significant reduction of blossom infection of 52% was found (Table 2). In a further control experiment on the highly susceptible Cotoneaster varity *C. salicifolius* the control effect could be confirmed with 85%.

Table 2. Fire Blight control with BioZell-2000B on apple variety Boskoop after natural blossom infection, 2001

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of Blossom clusters</th>
<th>Infection (%)</th>
<th>Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Infected</td>
<td></td>
</tr>
<tr>
<td>Inf.control</td>
<td>720</td>
<td>70</td>
<td>9.7 a</td>
</tr>
<tr>
<td>BioZell-2000B</td>
<td>680</td>
<td>30</td>
<td>4.7 b</td>
</tr>
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</table>

Figure 3. Disease index of fire blight on shoots of M26 rootstock after treatment with Bio-Zell-2000B

References


Efficacy of control agents on powdery mildew: a comparison between two populations

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² SafeCrop Centre, Istituto Agrario di S. Michele all’Adige, via Mach 1, S. Michele all’Adige, TN, 38010, Italy, email: ilaria.pertot@ismaa.it;
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Abstract: Powdery mildew (Sphaerotheca macularis f.sp. fragariae) causes severe losses in strawberries. The aims of the study were to test several alternatives to chemical fungicides of strawberry powdery mildew and to compare two populations of S. macularis, one from a temperate climate (Northern Italy) and the other form a Mediterranean climate (Israel) in their sensitivity to the control agents. A wide selection of BCAs (Ampelomyces quisqualis, Trichoderma harzianum T39, Bacillus subtilis, Bacillus sp. F77, Cladosporium tenuissimum), chemical fungicides (azoxystrobin, kresoxim methyl, penconazole, hexaconazole, triaconazole, triadimenol, nuarimol, fenarimol, pyraclostrobin + nicobifen, mancozeb + famoxadon, pirifeno, pirazofos, polyoxin Al, sulphur, sulphur + pinolene, ultrafine oil), plant extracts (Milsana – an extract of giant knotweed, neem oil, Agribioprop – a plant extract), resistance inducers (acylbenzolar-s methyl, harpin), salts and mineral nutrients (potassium bicarbonate, sodium bicarbonate, potassium phosphate monobasic, calcium carbonate, iron chelate, mineral nutrients solution) were tested in conidial germination tests. Several control agents were more effective on leaves than on glass (acylbenzolar-s methyl, harpin, T. harzianum T39 and Bacillus sp. F77). Salts better influenced germ tube elongation than germination. All the chemical fungicides were effective in inhibition of germination and/or the rate of germ tube elongation. The chemicals that had no significant effect on germination were tetraconazole and triadimenol, but they reduced germ tube elongation. Kresoxim methyl was the only chemical that had a different effect in Italy if compared with Israel, probably because of the presence of resistant strains in Israel and not in Italy, where it is not yet registered for the use on strawberry. The role of the plant tissue in the mechanism of action of some biocontrol agents and resistance inducers can be hypothesised. Although RAPD PCR analysis of genomic DNA with arbitrary primers and apPCR with primers specific to microsatellites show that pools of isolates randomly collected in Italy and Israel were different, the Italian and Israeli S. macularis populations can be considered similar in sensitivity to the tested control agents, except for chemicals that are already known to select resistant strains.

Introduction

Powdery mildew (Sphaerotheca macularis f.sp. fragariae) (Maas, 1998; Spencer, 1978) readily infected strawberry leaves and fruits. It causes severe losses in strawberries both in traditional cropping systems in Mediterranean climates and in greenhouse soil free systems, which are more common in central Europe and in some areas of Northern Italy. Most fungicide sprays applied to strawberry are targeted at control of powdery mildew. However, routine sprays are no longer acceptable and only high quality fruit can be marketed. Alternative approaches to disease control are aimed at developing integrated disease management that include non-chemical fungicides and biocontrol agents. No detailed information is available about the pathogen or its control.
The aims of the study were to test several alternatives to chemical fungicides of strawberry powdery mildew and to compare two populations of *S. macularis*, one from a temperate climate (Northern Italy) and the other from a Mediterranean climate (Israel) in their sensitivity to the control agents.

**Methods**

The selection of control agents is detailed in Table 1. The list included BCAs (*Ampelomyces quisqualis, Trichoderma harzianum T39, Bacillus subtilis, Bacillus sp. F77, Cladosporium tenuissimum*), chemical fungicides (azoxystrobin, kresoxim methyl, penconazole, hexaconazole, miclobutanil, tetraconazole, triadimenol, nuarimol, fenarimol, pyraclostrobin + nico-bifen, mancozeb + famoxadon, pirifenox, pirazofos, polyoxin Al, sulphur, sulphur + pinolene, ultrafine oil), plant extracts (Milsana – an extract of giant knotweed, neem oil, Agribioprop – a plant extract), resistance inducers (acylbenzolar-s methyl, harpin), salts and mineral nutrients (potassium bicarbonate, sodium bicarbonate, potassium phosphate monobasic, calcium carbonate, iron chelate, mineral nutrients solution) were tested in conidial germination tests.

The rate of germination and germ tube length were measured on glass slide and strawberry leaves. Biomass of the hyphae produced by the germinated conidia was calculated. The comparison of the two populations (Israeli and Italian) regarding several parameters (germination, biomass and germ tube length) was carried out in respect to susceptibility of the powdery mildew to a selection of the listed control agents. The control results related to the different control agents were grouped according to the nature of the agents.

apPCR with primers specific to Micro satellites and RAPD PCR with random primers was used with genomic DNA of Italian (IT) and Israeli (IS) pools of isolates.

<table>
<thead>
<tr>
<th>#</th>
<th>Active ingredient</th>
<th>Commercial name</th>
<th>Company</th>
<th>a.i. (%)</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Ampelomyces quisqualis</em> AQ10</td>
<td>Intrachem-bio Italia (IntBioIt)</td>
<td>BCA 58</td>
<td>0.08 g/l</td>
<td></td>
</tr>
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<td>2</td>
<td><em>Ampelomyces quisqualis</em> AQ10</td>
<td>IntBioIt</td>
<td>BCA 58</td>
<td>0.04 g/l</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Ampelomyces quisqualis</em> AQ10 + Ultrafine oil</td>
<td>UFO IntBioIt</td>
<td>BCA 58</td>
<td>0.08 g/l</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>T. harzianum T39</em></td>
<td>Trichodex</td>
<td>Makhteshim</td>
<td>BCA 20</td>
<td>0.20 g/l</td>
</tr>
<tr>
<td>5</td>
<td><em>T. harzianum T39</em></td>
<td>Trichodex</td>
<td>Makhteshim</td>
<td>BCA 20</td>
<td>4.00 g/l</td>
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<tr>
<td>6</td>
<td><em>Bacillus subtilis</em></td>
<td>Serenade</td>
<td>IntBioIt</td>
<td>BCA</td>
<td>4.00 g/l</td>
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<tr>
<td>7</td>
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<td>CNR-FI</td>
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<td>Neemgard</td>
<td>Agron (Israel)</td>
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<td>Ophir</td>
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<td>Kaken Pharm.</td>
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<td>12</td>
<td>Pyraclostrobin + Nicobifen</td>
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<td>BASF-AGRO</td>
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<td>26.7</td>
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<td>Sulphur</td>
<td>(Gofritar)</td>
<td>(Elf Atochem)</td>
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<td>Microthiol</td>
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<td>Domark combi</td>
<td>ISAGRO CHE</td>
<td>1+40</td>
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<td>Domark</td>
<td>ISAGRO CHE</td>
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<td>1.00 ml/l</td>
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<tr>
<td>21</td>
<td>Mancozeb+Famoxadon</td>
<td>Clipman</td>
<td>Du Pont CHE</td>
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<td></td>
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<td>22</td>
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<tr>
<td>23</td>
<td>EDDHA iron</td>
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<td>30</td>
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<tr>
<td></td>
<td>+ Ultrafine oil</td>
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<td>IntBioIt CHE</td>
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<td>35</td>
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<td>36</td>
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<td>38</td>
<td>K phosphate monobasic</td>
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<td>99</td>
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<tr>
<td></td>
<td>Monobasic</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>39</td>
<td>K phosphate monobasic</td>
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<tr>
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<td>Monobasic</td>
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<td>Agribioprop Tricoderma NSB</td>
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<td>extracts</td>
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<td>Syngenta</td>
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<td>0.64 g/l</td>
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<td>45</td>
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<td>Oxilyzer</td>
<td>CBC Europe NSB</td>
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<tr>
<td>46</td>
<td>Untreated</td>
<td>Water</td>
<td>NNN</td>
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</tbody>
</table>

BCA=biocontrol agent, CHE=chemical, OTH=other, NSB=natural substance, SAL=salt
Results

Several control agents were more effective on leaves than on glass (acylbenzolar-s methyl, harpin, *T. harzianum* T39 and *Bacillus* sp. F77). Salts better influenced germ tube elongation than germination. All the chemical fungicides inhibited germination and/or the rate of germ tube elongation. The chemicals that had no significant effect on germination were tetraconazole and triadimenol, but they reduced germ tube elongation. Kresoxim methyl was the only chemical that had a different effect in Italy and Israel, probably because of the presence of resistance Israel and not in Italy, where it is not yet registered for the use on strawberry (Figures 1- 4).

![Germination and Hyphae Biomass](image1.png)

Figure 1. Effect of various salts and oil on germination of *S. macularis* conidia on leaves

![Azoxyostrobin](image2.png)

Figure 2. Effect of fungicides on *S. macularis* originating from different regions

![Azoxyostrobin](image3.png)

Figure 3. Effect of fungicides on *S. macularis* germination on leaves
Figure 4. Effect of resistance inducers and other substances (upper row) and of biocontrol agents (lower row) on *S. macularis* germination on leaves (left) and glass slides (right)

Conclusions

Although RAPD PCR analysis of genomic DNA with arbitrary primers and apPCR with primers specific to micro-satellites show that pools of isolates randomly collected in Italy and Israel were different, the Italian and Israeli *S. macularis* populations can be considered similar in sensitivity to the tested control agents, except for chemicals that are already known to select resistant strains.

Acknowledgements

The authors kindly acknowledge Salvatore Moricca IPAF, CNR Firenze, for providing the *Cladosporium tenuissimum* strain. The research was funded by Fondo per la ricerca, Provincia Autonoma di Trento, SAFECROP project (tests on the Italian powdery mildew population).

References

Grapefruit extract inhibits sporulation and development of *Phytophthora ramorum* on *Rhododendron*

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email: lorlikow@insad.pl

Abstract: *In vitro* activity of grapefruit extract and chitosan in the inhibition of *Phytophthora ramorum* sporulation and their efficacy in the control of leaf and stem rot development was evaluated. Amendment of soil leachate with grapefruit extract resulted in the inhibition of pathogen sporulation. Zoosporangia were more susceptible to the extract than chlamydospores. Chitosan only slightly inhibited zoosporangia formation. Spraying of *Rhododendron* inoculated with *P. ramorum* with grapefruit extract at conc. A concentration of 165 µg/cm³ inhibited 2-3 times the spread of necrosis on stems and leaves. Pre-and post-inoculation spraying of plants with chitosan at concentration of 1000 µg/cm suppressed the disease spread about 40%.

Keywords: biocontrol, *Rhododendron* blight, sporulation, leaf, stem, necrosis, inhibition

Introduction

*Phytophthora ramorum* is the causal agent of sudden oak death in California USA, twig blight of *Rhododendron* and shoot rot of *Viburnum* spp. in Europe and the USA (Rizzo et al., 2002; Werres et al., 2001). Actually more than 40 species of host plants are known and every year new hosts are discovered. In Poland *P. ramorum* was detected the first time on diseased twigs of *Rhododendron* in 2000 (Orlikowski & Szkuta, 2002). The pathogen zoosporangia are easily dispersed with wind, water sometimes on a long distance from the disease plants. The spread of that species from ornamental nurseries, garden, parks and other plant arrangements to forests are especially dangerous. In such case besides removing and burning of disease plants chemical and biological control of *P. ramorum* is necessary. Spraying of plants with fosetyl Al, its mixture with fenamidone, oxadiroxyl suppressed the formation of zoosporangia and chlamydomspores on inoculated leaf blades and stems of *Rhododendron*. Additionally chemicals suppressed the development of leaf and stem rot (Orlikowski, 2004). In this study, *in vitro* influence of grapefruit extract on *P. ramorum* colony growth, zoosporangia and chlamydomspore formation and development of necrosis on *Rhododendron* leaves and stems were evaluated.

Materials and methods

*Phytophthora ramorum*, isolate RH6 from diseased *Rhododendron* was used. Stock culture was maintained on V8 juice agar at 20°C. For the pathogen control Biosept 33 SL (33% of grapefruit extract) supplied by Cintamani Poland and Biochikol 020 PC (2% chitosan) produced by Gumitex Poly-Farm, Poland were used. Formation of zoosporangia and chlamydomspores in the presence of biocontrol agents was evaluated in soil leachate. Procedure described by Orlikowski (2001) was used. In greenhouse trials *Rhododendron* cv. Nova Zembla leaves and stem tops were inoculated with 3 mm diam plugs of *P. ramorum* taken from culture grown 10 days on V8 juice agar. After 12 h plants were sprayed once or twice
with grapefruit extract at concentrations of 165 and 330 µg/cm³. Chitosan at concentration of 1000 µg/cm³ was applied one week before inoculation and 12 h after inoculation. After treatment plants were covered with plastic tunnel to keep moisture above 92% and temperature varying from 15º to 21ºC. One and two weeks after the second spraying length of necrosis on stems and diam of lesions on leaf blades were measured. Experimental design was completely randomised with four replications (one Petri dish and five plants in each replicate). Trials were repeated at least twice with three week interval.

**Results and discussion**

*Sporulation of P. ramorum in the presence of biocontrol agents*

In soil leachate amended with 8 µg of grapefruit extract (GE)/cm³ zoosporangia formation on infected leaf disks was drastically inhibited (Table 1). Their formation were only sporadically observed in medium containing 40 µg of GE/cm³. Inhibition of chlamydospore production on infected leaf disks were observed in the medium containing 200 µg of GE/cm³ (Table 1). Observation of leaf disks under microscope showed that already at 8 µg of GE/cm³ zoosporangia were formed only singly but not in clusters. They were longer than in nonamended medium. At 40 µg/cm³ disintegration of hyphae was observed. Chitosan applied at doses 200 and 1000 µg/cm³ inhibited the colony growth of the pathogen about 20% but did not influence sporangia formation.

<table>
<thead>
<tr>
<th>Grapefruit extract (µg/cm³)</th>
<th>Incubation time (h)</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>0</td>
<td>16 b 0.4 a</td>
<td>51 c 0.6 a</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.7 a 5.5 b</td>
<td>1.4 a 7.0 c</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.6 a 7.0 c</td>
<td>0.6 a 8.0 c</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0 a</td>
<td>0 a</td>
<td></td>
</tr>
</tbody>
</table>

Means followed by the same letter, do not differ with 5% of significance (Duncan’s multiple range test).

*Development of Rhododendron blight in greenhouse trials*

Grapefruit extract in both concentrations suppressed the spread of necrosis on stems and leaf blades (Table 2). Spraying of plants with GE at concentration of 330 µg/cm³ twice was more effective than one treatment. Oxadixyl, as the standard fungicide, applied once was more effective than GE (Table 2). One application of chitosan 12 h after inoculation with *P. ramorum* only slightly suppressed the spread of necrosis on stems and leaves. Spraying of *Rhododendron* seven days before inoculation and 12 h after inoculation decreased the spread of necrosis on stems about 40% (Table 2).

Inhibition of growth and sporulation of *P. cryptogea* by grapefruit extract disintegration of hyphae and deformation of zoosporangia was observed by Orlikowski (2001). Additionally, the product caused significant decrease of *Phytophthora* root and stem rot development of Lawson cypress, yew and heather (Orlikowski, 2002). Present trials showed
that grapefruit extract may completely inhibit zoosporangia formation but not chlamydospores of *P. ramorum* so in the control of the disease. In such situation repetition of plant treatment with both products combined with pruning of diseased parts of *Rhododendron* are necessary.

Table 2. Biological activity of grapefruit extract and chitosan in the control of *Phytophthora ramorum* spread on *Rhododendron*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Grapefruit extract (µg/cm³)</th>
<th>Number of sprayings</th>
<th>Necrosis length/diam (mm) 7 days after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>stems</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>45.6 d</td>
</tr>
<tr>
<td>Grapefruit extract</td>
<td>165</td>
<td>1</td>
<td>22.3 b</td>
</tr>
<tr>
<td></td>
<td>165</td>
<td>2</td>
<td>22.3 b</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>1</td>
<td>30.0 c</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>2</td>
<td>22.8 b</td>
</tr>
<tr>
<td>Chitosan</td>
<td>1000</td>
<td>1</td>
<td>29.6 c</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>2</td>
<td>31.2 c</td>
</tr>
<tr>
<td>Oxadixyl</td>
<td>160</td>
<td>1</td>
<td>4.5 a</td>
</tr>
</tbody>
</table>

Means followed by the same letter, do not differ with 5% of significance (Duncan’s multiple range test).

References


Comparison of wood colonisation by local *Phlebia gigantea* strains, Rotstop® and *Trichoderma viride* on spruce logs in Alpine environment

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**Abstract:** Biological control of *Heterobasidion* root rot is a common silvicultural procedure in North Europe. One of the most commonly applied protectants is Rotstop®, a commercial preparation based on a *Phlebia gigantea* strain isolated from Finland. In previously conducted field tests in the Alps, Rotstop® has performed quite well as a biological control agent of *H. annosum* in spruce forest stands. Because of the specific environmental and ecological characteristics of the Alps region, the suitability and adaptability of selected local strains of *P. gigantea* was now compared with Rotstop® and other fungi used for biological control. The field experiment was carried out in a pure Norway spruce (*Picea abies*) stand in Trentino, North-Eastern Italian Alps. Rotstop®, six Italian strains of *P. gigantea* (four from Trentino, one from Central Italy and one from Southern Italy), and two strains of *Trichoderma viride* (one from Finland, one from Tuscany) were applied onto healthy spruce logs. A urea treatment was used as a reference. Wood disks were cut from the logs after two growing seasons in autumn 2001. The number of colonies of all antagonistic fungi and the percentage of wood infected were determined from both sides of the disks. Among the treatments, Rotstop® proved to be the second best in terms of both the number of colonised logs and the percentage of colonised surface. *P. gigantea* strains from Trentino showed variable ability to colonise spruce wood, with one strain showing significantly higher wood colonisation than Rotstop®. Among the Italian *P. gigantea* strains, the ones from Trentino showed significantly higher wood colonisation than the strain from South Italy. In all cases, *P. gigantea* strains colonised most of the sapwood and occasionally also the hardwood. The Italian *Trichoderma* strain gave results comparable to those obtained with the *P. gigantea* strains from Trentino. These data indicate that there is considerable variation in the wood colonisation ability among strains belonging to the same species. The impact of biodiversity of antagonistic strains on the control of *H. annosum* is discussed.

**Introduction**

Root rot caused by *Heterobasidion annosum* is a great problem also in the Alpine conifer forests. Damages on the trees are more serious in plantations than in old forests, and usually appear after the first thinning operation. Primary infection of a Norway spruce stand by *H. annosum* takes place through fresh thinning stumps or wounds produced on roots and at the base of the stem. Basidiospores landing on these entrance points give rise to mycelia, which colonizes the root systems. The secondary spread in the site takes place as mycelial growth through root-to-root contacts to adjacent trees (see e.g. Stenlid & Redfern, 1998). It has also been shown that *H. annosum* remains viable in stumps for decades after cutting and that this stump inoculum is the major source of infection in the new tree generation (Piri, 1996). Biological control of *Heterobasidion* root rot is a common procedure in North European countries. One of the most applied protectant against *H. annosum* infection is *Phlebia gigantea* (Holdenrieder & Greig, 1998). This saprophytic fungus has several characteristics
that make it suitable as a biological control agent: a) like *H. annosum*, *P. gigantea* colonises rapidly fresh conifer stumps and is antagonistic to *H. annosum*; b) it produces asexual spores in pure culture, which makes it relatively easy to produce a spore solution for stump treatment; c) the fungus is not allergenic. Because of these favourable traits, the commercial preparation Rotstop® based on a selected Finnish *P. gigantea* strain, was issued to the market by a Finnish company Kemira OY (Korhonen et al., 1994).

In previous field tests in the Alps, Rotstop® performed quite well as a biological control agent of *H. annosum* in Norway spruce (*Picea abies* [L.] Karst.) forest stands (Holdenrieder & Greig, 1998). In Trentino, as in most temperate coniferous forests, *P. gigantea* is a common inhabitant of pine and spruce stumps, fruiting abundantly during warm rainy autumns. The strains growing on spruce have been proved to be interfertile with those growing on pine (Korhonen & Kauppila, 1988). A rapid increase in the number of sites infected by *Heterobasidion* root rot in Italy, together with new requirements for “environmentally-safe” pesticides, has stimulated research into potential biological control agents to prevent the spread of this pathogen. The principal aim of this study was to compare Rotstop® and local *P. gigantea* strains in their wood-colonising ability of spruce in Alpine environment.

**Material and methods**

As a first step, the growth rate of 16 heterokaryotic *P. gigantea* isolates were compared on agar medium. Six most vigorous strains were selected for the field test. The experiment was carried out in Trentino, North-Eastern Italian Alps, on a pure stand of Norway spruce (*Picea abies*) by using healthy spruce logs. Four healthy spruce stems with a stump diameter of ca. 20 cm were selected from a site free from *Heterobasidion* root rot and transferred to the experimental forest. The basal part of each stem was cut to 20-cm-long sections, 30 pieces being obtained from each stem. The cutting was performed within 3 hours before treatments. After cutting, the upper surface was divided in two symmetrical halves; one half was sprayed with the protecting agent, while the other half was covered. One to two hours later the whole surface was sprayed with sterile water. The protecting agents were: 1) urea 30 %, 2) *Trichoderma viride* 94268 (isolated from pine wood in Vantaa, South Finland), 3) Rotstop®, 4) *P. gigantea* 97092 (Italy, Trentino), 5) *P. gigantea* 97097 (Italy-Trentino), 6) *P. gigantea* 97099 (Italy-Trentino), 7) *P. gigantea* 97104 (Italy-Trentino), 8) *P. gigantea* 95051 (Italy-Tuscany Tirrenia), 9) *P. gigantea* 95042 (Italy-Abruzzo), 10) *T. viride* (Italy). The conidia concentrations were adjusted to c. 5.000 spores/ml for each *P. gigantea* strain (including Rotstop®) and to c. 20.000 conidia/ml for *Trichoderma*. The number of repetitions was 12 (3 repetitions in each spruce stem).

After treatment, the stem pieces (spatially randomised) were left standing in a humid place in the forest (Figure 1). The weather during the treatment was sunny and dry, and the six following days also were rainless. The stem pieces and the soil around them were sprayed with clean water every week during the dry season. In autumn 2001, two growing seasons after the treatment, wood disks were cut from the logs and bark removed. Two discs were cut; the first disc (3 cm thick) was discarded, the second disc (5 cm thick) was mechanically planed, to smooth down the cut surface, and then washed in running water.

After an incubation of 7 days in plastic bags at room temperature, the discs were investigated on both sides for the presence of *P. gigantea* and *T. viride*. Also the area occupied by *P. gigantea* was approximately determined on the basis of orange-brown colour characteristic to wood colonised by the fungus. The efficacy of the treatment was calculated by comparing the area occupied by *P. gigantea* and *T. viride* on treated and untreated halves of each disc surface.
Results and discussion

The number of colonies of all antagonistic fungi and the percentage of wood infected was counted on both sides of the disks. There was a high correlation in the colonisation between the two sides of each disc (3 and 8 cm from the treated surface). Practically no \textit{P. gigantea} was observed in the halves treated with urea, while moderate colonisation levels were observed in the untreated halves of this treatment (Figure 2). Among the fungal treatments, Rotstop\textsuperscript{®} proved to be the second best in the area colonised by \textit{P. gigantea}. This was previously found also by La Porta et al. (2001; 2002). The \textit{P. gigantea} strains from Trentino showed a variable degree of capacity to grow in spruce wood, but one \textit{Phlebia} strain from Trentino performed significantly better than Rotstop\textsuperscript{®}. In all other treatments \textit{P. gigantea} from Italy showed lower performance in terms of colonised wood, although, in general, strains from Trentino showed more efficient wood colonisation than strains from Central and Southern Italy. In all cases \textit{P. gigantea} colonised most of the sapwood and sometimes also the hardwood. The Italian \textit{Trichoderma} treatment resulted, both in the untreated and treated halves, in a \textit{P. gigantea} colonisation level comparable to those obtained with \textit{P. gigantea} isolates from Trentino. The Finnish \textit{T. viride} treatment, on the other hand, resulted in a relatively low \textit{P. g.} colonisation in the treated halves while comparably high \textit{P. gigantea} colonisation levels were observed in the untreated halves. These observations indicate colonisation by indigenous \textit{P. gigantea} strains in the \textit{Trichoderma} treatments. The low \textit{P. gigantea} colonisation level in the halves treated with the Finnish \textit{Trichoderma} could suggest that the strain used is antagonistic to \textit{P. gigantea}.

Marked differences in the colonisation area of \textit{P. gigantea} were now observed between logs treated with Rotstop\textsuperscript{®} and different \textit{P. g.} isolates. As the experiment was carried out in field conditions in an environment with an indigenous \textit{P. gigantea} population, colonisation by natural \textit{P. g.} strains can interfere with the results now presented. Somatic compatibility tests are needed to confirm if and how significantly indigenous \textit{P. g.} strains contribute to the colonisation data in the treatments where various \textit{P. g.} strains and Rotstop\textsuperscript{®} were artificially introduced. Keeping this caution in mind, the data does suggest that there is genetic variation in the wood colonisation ability between different strains of \textit{P. gigantea}. Maximum \textit{P. gigantea} colonisation was now observed in the treatment with the Trentino’s isolate P.g.
97097, while application of the Abruzzo’s isolate gave the poorest colonisation levels. Rapid and effective wood colonisation by *P. gigantea* may be a significant factor in a successful control of the spreading of *H. annosum*.

![Figure 2. Wood colonised by *P. gigantea* on the treated and non-treated halves of the logs](image)

The high degree of polymorphism and heterozygosity observed among different populations of these species markers suggests that the amount of genetic variation within this fungus is considerable. The lack of distinct groups of similar banding patterns supports the idea of *P. gigantea* being a true biological species consisting of a single intersterility group. (Vainio et al., 1998). In addition, it was proved that the survival time of *P. g.* was longer in Norway spruce stumps compared to Scots pine as no isolates were obtained from pine stumps 6 years after treatment, whereas in about half of the spruce stumps the fungus was still present. However, also in Norway spruce, Rotstop® seems do not cause any immediate threat to the genetic diversity of natural population of *P. gigantea* (Vainio et al., 2001). Nevertheless, application of fast-colonising local strains for biological control in a particular ecosystem, e.g. the Alps region, could be recommended as these local strains are adapted to the prevailing conditions. Furthermore, application of local strains would not interfere with the dynamics of the indigenous populations of *P. gigantea*. Further studies on screening *P. gigantea* strains from the Alps region for wood colonisation ability and efficiency as a biocontrol agent can be justified.

**Acknowledgements**

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References


Selectivity and effectiveness evaluation of electrolyzed acidic water (EAW) alone and in mixture with Shin-Etsu surfactants, against tomato late blight Phytophthora infestans

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Abstract: Electrolyzed Acid Water (EAW), generated by electrolysis from tap water and potassium chloride (KCl), is a mean of high level disinfection similar to that already used in the medical sector for endoscopes reprocessing. Thanks to the extremely high efficacy on a wide range of fungi, bacteria and viruses the possibility of its application in agriculture should be investigated for the reduction of conventional chemical treatments. With this aim, first laboratory trials were carried out in 2003, applying EAW, alone and in mixture with Shin-Etsu surfactants, in order to assay its preventive (2003/EAW/01, 02) and curative fungicide activity (2003/EAW/03, 04, 05, 06, 07, 08) against Phytophthora infestans, the causal agent of tomato late blight. The commercial standard fungicides compared were copper-based.

Key words: electrolysed acid water, Phytophthora infestans, late blight, tomato

Introduction

Electrolyzed Acidic Water (EAW), generated by electrolysis from tap water and potassium chloride (KCl), is a mean of high level disinfection similar to that already used in medical sector for endoscopes reprocessing. Thanks to the extremely high efficacy on a wide range of fungi, bacteria and viruses the possibility of its application in agriculture should be investigated for the reduction of conventional chemical treatments. With this aim, first laboratory trials were carried out in 2003, applying EAW, alone and in mixture with Shin-Etsu surfactants, in order to assay its preventive (2003/EAW/01, 02) and curative fungicide activity (2003/EAW/03, 04, 05, 06, 07, 08) against Phytophthora infestans, the causal organism of tomato late blight. The commercial standard fungicides compared were copper-based.

Materials and methods

Evaluation of preventive activity
Once treated, plants were let to dry for almost 1 hour. After this interval, they were artificially inoculated. Immediately after the inoculum, plants were put in a climatic chamber with constant temperature (24°C) and relative humidity about 97% in order to allow the pathogen to penetrate.

Evaluation of curative activity
Once artificially inoculated, plants were put in a humid chamber for almost 2 hours in order to allow the pathogen to penetrate. In the next phase plants were treated according to the protocol scheduling. Once dried, plants were put in a climatic chamber with constant temperature (24°C) and relative humidity about 97% in order to allow the developing of the disease.
At sporulation, normally 3-4 days after treatment, assessment was done consisting on the evaluation of the percentage of leaf surface infected of 5 compound leaves for each plant. Data were analyzed by analysis of variance ANOVA and means were compared using the Least Significant Difference test (LSD).

**Trials specifications**

Target pathogen: *Phytophthora infestans*; crop: tomato, var. Marmande; trial conditions: climatic chamber with constant temperature (24°C) and relative humidity about 97% to permit pathogen sporulation; number of replicates: 3 plants of tomato for each variant; number of treatments: 1 treatment with hand sprayer until runoff; artificial inoculums: *Phytophthora infestans* propagula (20.000 propagula/ml); assessment: evaluation of percent of infected leaf surface according to EPPO guideline PP1/31(2), 1997.

**Results**

Tables 1 and 2 summarize the results obtained from the trials concerning the evaluation of fungicide preventive activity of EAW with an application, respectively, 1 and 5 hours before the artificial inoculum. For each timing, EAW was mixed with two Shin-Etsu surfactants: KF642 e KF643 at 0.1%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active ingredient</th>
<th>Infected leaf surface (%)</th>
<th>Efficacy (%)</th>
<th>Phytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>64.7 b</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
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<td>77</td>
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<tr>
<td>EAW + KF643</td>
<td>EAW + KF643</td>
<td>6.7 a</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>Kocide 2000</td>
<td>Copper hydroxide</td>
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<td>99</td>
<td>0</td>
</tr>
<tr>
<td>Pasta Caffaro Blu</td>
<td>Copper oxychloride</td>
<td>0.7 a</td>
<td>99</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active ingredient</th>
<th>Infected leaf surface (%)</th>
<th>Efficacy (%)</th>
<th>Phytotoxicity</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
<td>–</td>
<td>93.3 c</td>
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<td>0</td>
</tr>
<tr>
<td>EAW + KF642</td>
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<td>42.0 b</td>
<td>55</td>
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<tr>
<td>EAW + KF643</td>
<td>EAW + KF643</td>
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<td>81</td>
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<tr>
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<td>Copper hydroxide</td>
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<td>93</td>
<td>0</td>
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<tr>
<td>Pasta Caffaro Blu</td>
<td>Copper oxychloride</td>
<td>14.0 a</td>
<td>85</td>
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</tbody>
</table>

Tables 3, 4 and 5 summarize results obtained from the trials concerning the evaluation of fungicide curative activity of EAW with an application respectively 3, 7 and 10 hours after the artificial inoculum. For each timing, EAW was used alone and mixed with two Shin-Etsu surfactants: KF642 e KF643 at 0.1%.
Table 3. Treatment three hour after the artificial inoculum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active ingredient</th>
<th>Infected leaf surface (%)</th>
<th>Efficacy (%)</th>
<th>Phytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>50.0 b</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>EAW + KF642</td>
<td>EAW + KF642</td>
<td>42.0 ab</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>EAW + KF643</td>
<td>EAW + KF643</td>
<td>36.7 ab</td>
<td>27</td>
<td>0</td>
</tr>
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<td>EAW</td>
<td></td>
<td>34.7 a</td>
<td>31</td>
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<tr>
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<td>Copper oxychloride</td>
<td>46.7 ab</td>
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<td>0</td>
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<tr>
<td>Forum R</td>
<td>Dimetomor+ +copper</td>
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<td>82</td>
<td>0</td>
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</table>

Table 4. Treatment seven hour after the artificial inoculum

<table>
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<th>Treatment</th>
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<th>Infected leaf surface (%)</th>
<th>Efficacy (%)</th>
<th>Phytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>60.7 c</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>EAW + KF642</td>
<td>EAW + KF642</td>
<td>26.7 ab</td>
<td>56</td>
<td>0</td>
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<td>EAW + KF643</td>
<td>11.3 a</td>
<td>81</td>
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<tr>
<td>EAW</td>
<td></td>
<td>18.0 a</td>
<td>56</td>
<td>0</td>
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<tr>
<td>Pasta Caffaro Blu</td>
<td>Copper oxychloride</td>
<td>46.7 bc</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Forum R</td>
<td>Dimetomor+ +copper</td>
<td>0.0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5. Treatment ten hour after the artificial inoculum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active ingredient</th>
<th>Infected leaf surface (%)</th>
<th>Efficacy (%)</th>
<th>Phytotoxicity</th>
</tr>
</thead>
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<td>61.3 c</td>
<td>–</td>
<td>0</td>
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<tr>
<td>EAW + KF642</td>
<td>EAW + KF642</td>
<td>24.7 ab</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>EAW + KF643</td>
<td>EAW + KF643</td>
<td>16.7 ab</td>
<td>73</td>
<td>0</td>
</tr>
<tr>
<td>EAW</td>
<td></td>
<td>11.3 a</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>Pasta Caffaro BLU</td>
<td>Copper oxychloride</td>
<td>22.0 ab</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Forum R</td>
<td>Dimetomor+ +copper</td>
<td>2.0</td>
<td>97</td>
<td>0</td>
</tr>
</tbody>
</table>

Conclusion

The assay showed that EAW alone and in mixture with the Shin-Etsu organo-silicone KF643 was highly effective for tomato late blight control, both as preventive and curative treatment. Its effectiveness is comparable to copper-based formulations. EAW curative activity shows its effectiveness in an interval ranging from 3 to 10 hours after inoculation, thus proving to be particularly useful for early post-inoculation applications. EAW preventive activity shows its
effectiveness in an interval ranging from 1 to 5 hours before inoculation, thus enabling
defensive applications before the foreseeable infecting rain. After 24 hours, once the pathogen
has penetrated the plant, EAW + surfactant is no more effective. No leaf burn or other
symptom of phytotoxicity were observed for any of the tested products during the trial.

As this preliminary assay has been carried out under artificial conditions, it will be
necessary to verify the results under field conditions. However, the results are encouraging,
considering that the trial was carried out under conditions particularly suitable for the disease
development. As far as future commercial applications are concerned, the main difficulty lies
in the disease control in case of long intervals between disease promoting conditions (rain)
and the timing of application. This circumstance frequently occurs in open field horticulture,
where the re-entry interval after heavy rain events is very long, due to the heavy equipment
used and to lack of grass-cover between rows. The utilization of EAW for protected crops or
other crops such as grape growing, where grass covering between rows is present, could be
very interesting, because none of the previous negative conditions would occur.

The adoption of EAW for soil disinfestations could represent another possibility to take
into consideration thanks to the presence of chlorine that is effective against soil
pathogens. Also in this case the application under greenhouse conditions could be easier and
more practical due to the lower quantity of product needed.

Acknowledgement

An acknowledgement to Shin-Etsu Chemical Company Ltd. that provided the surfactants for
the trials.
Biological control of powdery mildew by Q-fect WP
(*Ampelomyces quisqualis* 94013) in various crops

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Abstract: An isolate, *Ampelomyces quisqualis* 94013, was selected as a biocontrol agent for the control of powdery mildew in various crops. It was formulated as a wettable powder and termed Q-fect WP by Green Biotech Co. Ltd. in Korea. The Q-fect WP was treated onto 8 plant species to control powdery mildew in the greenhouse. The occurrence of powdery mildew in the crops was evaluated 7 days after treatment. The control value of powdery mildew by the commercial product was 67.7% in strawberry, 75.2% in cucumber, 74.8% in melon, 88.9% in tomato, 86.2% in *Ligularia fischeri*, 77.1% in gerbera, 68.5% in rose, and 70.0% in grape. The results showed that Q-fect WP is effective as a biofungicide for controlling powdery mildew in the eight crops. The technical grade of *Ampelomyces quisqualis* 94013 was registered as a biofungicide of cucumber powdery mildew in March 2003, and the commercial product was granted in Korea in April 2004.

Key words: *Ampelomyces quisqualis* 94013, biocontrol, biofungicide, powdery mildew, Q-fect WP

Introduction

Powdery mildew pathogens attack over 11,800 plant species world-wide and approximately 300 species are recorded as host plants in Korea (Shin, 2000). Powdery mildews are significantly important diseases since they cause severe damages on many economically important crops such as cucumber, oriental melon, pumpkin, tomato, strawberry, eggplant, red-pepper and rose (Shin, 1994). In general, the disease becomes epidemic under the favorable environmental conditions; warm and dry weather, poor air circulation, low light intensity, excessive nitrogen fertilization, continuous cropping and poor management due to shortage of labour (Spencer, 1978). Consequently, plants growing intensively under greenhouse condition are more vulnerable to the disease than those cultivated in the open fields. Control of the powdery mildew has mainly relied on agrochemicals. However, continuous use of chemicals has induced resistant strains of the pathogen and generated various negative side effects on environments (Nakazawa, 1995). In order to overcome these problems, biological control through useful microorganisms was attempted as a promising control measure to reduce the pathogen population and the disease severity (Hofstein, 1996; Falk et al., 1995).

The fungus, *Ampelomyces quisqualis* was the firstly reported as a hyperparasite of powdery mildew fungi by Yarwood in 1932, and subsequent researches found that the fungus parasites several genera of powdery mildew pathogens (Sztejngberg et al., 1985). In Korea, Lee (1976) firstly isolated the parasite from powdery mildew fungi on pumpkin and oriental melon, and Shin (1994) proposed that the hyperparasite could be a potential biocontrol agent
against powdery mildew. In this study, an isolate of the prospective hyperparasite, *A. quisqualis* 94013, was selected as a biocontrol agent against powdery mildew. It was formulated as a wettable powder and termed Q-fect WP by Green Biotech Co. Ltd. in Korea. Control effect of Q-fect WP was demonstrated against powdery mildew on plants of eight different crops in the greenhouse.

**Material and methods**

The Q-fect WP (*Ampelomyces quisqualis* 94013) formulated by Green Biotech Co. Ltd in Korea in 2000 was used for field tests. Its active ingredient was above 1x10⁷ spore/g of the formulation. The biofungicide was diluted to 500 to 1,000 times for foliar spray application onto plants in the greenhouse. The diluted solution of Q-fect WP was applied three times by 7-day-interval at early stage of powdery mildew development on eight crops in the greenhouse. Investigation of powdery mildew incidence was conducted 7 days after treatment of the solutions.

**Table 1. Suppression of powdery mildew by treatment with the Q-fect WP on eight different crops in the greenhouse**

<table>
<thead>
<tr>
<th>Crop</th>
<th>Treatment</th>
<th>Dilution (x)</th>
<th>Disease severity (%; plant part)</th>
<th>Control efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strawberry</td>
<td>Q-fect WP</td>
<td>500</td>
<td>5.1 a (fruit)</td>
<td>67.7</td>
</tr>
<tr>
<td></td>
<td>DBEDCEC (20%)</td>
<td>500</td>
<td>4.6 a</td>
<td>70.9</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>_</td>
<td>15.8 b</td>
<td>_</td>
</tr>
<tr>
<td>Cucumber</td>
<td>Q-fect WP</td>
<td>1,000</td>
<td>14.4 a (leaf area)</td>
<td>75.2</td>
</tr>
<tr>
<td></td>
<td>Fenarimol EC (12.5%)</td>
<td>4,000</td>
<td>8.7 a</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>_</td>
<td>58.0 b</td>
<td>_</td>
</tr>
<tr>
<td>Tomato</td>
<td>Q-fect WP</td>
<td>1,000</td>
<td>9.2 a (leaf)</td>
<td>88.9</td>
</tr>
<tr>
<td></td>
<td>Triflumizole WP (30%)</td>
<td>4,000</td>
<td>3.3 a</td>
<td>96.0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>_</td>
<td>82.9 b</td>
<td>_</td>
</tr>
<tr>
<td><em>Ligularia fischeri</em></td>
<td>Q-fect WP</td>
<td>1,000</td>
<td>13.3 a (leaf)</td>
<td>86.2</td>
</tr>
<tr>
<td></td>
<td>Azoxystrobin SC (30%)</td>
<td>3,000</td>
<td>4.3 a</td>
<td>95.6</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>_</td>
<td>96.7 b</td>
<td>_</td>
</tr>
<tr>
<td>Rose</td>
<td>Q-fect WP</td>
<td>1,000</td>
<td>14.4 a (leaf)</td>
<td>68.5</td>
</tr>
<tr>
<td></td>
<td>Fenarimol EC (12.5%)</td>
<td>4,000</td>
<td>8.7 a</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>_</td>
<td>58.0 b</td>
<td>_</td>
</tr>
<tr>
<td>Melon</td>
<td>Q-fect WP</td>
<td>500</td>
<td>19.0 a (leaf area)</td>
<td>74.8</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>_</td>
<td>75.5 b</td>
<td>_</td>
</tr>
<tr>
<td>Gerbera</td>
<td>Q-fect WP</td>
<td>500</td>
<td>8.0 a (leaf area)</td>
<td>77.1</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>_</td>
<td>35.0 b</td>
<td>_</td>
</tr>
<tr>
<td>Grape</td>
<td>Q-fect WP</td>
<td>1,000</td>
<td>12.6 a (fruit)</td>
<td>70.0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>_</td>
<td>42.0 b</td>
<td>_</td>
</tr>
</tbody>
</table>
Results and discussion

Incidence of powdery mildew caused by *Sphaerotheca aphanis* on fruits of strawberry was 15.8% in the control plots, 5.1% in the Q-fect WP-treated plots and 4.6% in the DBEDC EC-treated plots (Table 1). Severity of powdery mildew caused by *Sphaerotheca fusca* on leaves of cucumber was 58.0% in the control plots, 14.4% in the Q-fect WP-treated plots, and 8.7% in the Fenarimol EC-treated plots. Incidence of powdery mildew caused by *Golovinomyces cichoracearum* on leaves of tomato was 82.9% in the control plots, 9.2% in the Q-fect WP-treated plots, and 3.3% in the Triflumizole WP-treated plots. Incidence of powdery mildew caused by *S. fusca* on leaves of *Ligularia fischeri* (Ledeb.) was 96.7% in the control plots, 13.3% in the Q-fect WP-treated plots, and 4.3% in the Azoxystrobin SC-treated plots.

Incidence of powdery mildew caused by *Sphaerotheca pannosa* on leaves of rose was 71.1% in the control plots, 22.4% in the Q-fect WP-treated plots, and 6.7% in the Fenarimol EC-treated plots, respectively. Severity of powdery mildew caused by *S. fusca* on leaves of melon was 75.5% in the control plots, and 19.0% in the Q-fect WP-treated plots, respectively. Severity of powdery mildew caused by *S. fusca* on leaves of gerbera was 35.0% in the control plots, and 8.0% in the Q-fect WP-treated plots, respectively. Incidence of powdery mildew caused by *Uncinula necator* on fruits of grape was 42% in the control plots, and 12.6% in the Q-fect WP-treated plots, respectively.

A formulated product of *A. quisqualis* was registered in the late 1980s in Australia, but the high humidity requirements have hampered its efficacy. AQ10TM, developed by Ecogen Inc., was reported to tolerate lower relative humidities, and registered against grape powdery mildew in California, USA. But AQ10 has not been used for the control of powdery mildew in Korea. In conclusion, Q-fect WP may be used effectively as a biofungicide for controlling powdery mildew on various crops since it showed strong parasitism to six species of *Sphaerotheca* and one species of *Golovinomyces*, and *Uncinula* which cause serious damages in many crops in Korea.

References


Control of powdery mildew on organic pepper

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Abstract: Powdery mildew of pepper, caused by Leveillula taurica, is a disease affecting most commercial pepper cultivars grown in Israel. Disease symptoms include white powdery mycelial growth on the underside of the leaves, and, on the upper surface of the leaves, chlorotic spots that become necrotic. Affected leaves tend to drop off the plant, causing direct sun-scald damage to the fruits and reduced photosynthetic capacity of the plant. In organic management, sulfur-containing agents efficiently control the disease. However, since sulfur agents may harm the beneficial insects introduced into the greenhouse to control pests, our main objective in this study was to search for alternative control means. Experiments were carried out under full organic management in protected tunnels at Yair station. In 1999-2000, Fiesta and 107 cultivars were used, and in 2000-2001 Nibla and Parker. In the first experiment, sulfur agents either sprayed on the foliage or fumigated in the greenhouse were, as expected, very efficient in controlling the disease. They were better than the other tested treatments: water extract of cattle manure compost, Kaligrin (potassium bicarbonate) and Rifol (fish oil) (data not shown). In the second year, all treatments – including Neemgard, water extract of grape marc compost, AQ10 (Ampelomyces quisqualis), Kaligrin and Rifol – significantly reduced disease incidence compared to the non-treated control; however, disease incidence was lowest in plots treated with sulfur. Significantly higher yields were obtained in all treatments (especially in Neemgard), as compared with the control, but only in cultivar Nibla.

Key words: Leveillula taurica, biological control, neem-oil, potassium bicarbonate, mineral sulfur, compost water extract, Ampelomyces quisqualis

Introduction

Organic greenhouse pepper is an important crop in Israel, which during the last year increased to 25 ha. It is grown in insect-proof greenhouses or tunnels from September until April-May. Most (76%) of the organic production is for export to Europe (14,500 tons of organic vegetables, in 2002). Powdery mildew caused by Leveillula taurica (Lev.) Arm. is the most destructive pathogen of greenhouse and field-grown peppers in Israel (Reuveni & Rotem, 1973). The infection, in association with high temperatures, causes defoliation, which leads to smaller fruits because of the reduced photosynthetic area, and damage by sun scald. As chemical control is not possible under organic management, and since commercially acceptable resistant cultivars are not available, although genetic background of resistance to the pathogen was investigated (Daubeze et al., 1995), alternative methods of protection are essential. The common method of controlling powdery mildew on organic pepper with sulfur agents may harm beneficial insects introduced into the greenhouse to control pests (such as thrips). The main objective in this study was to search for alternative means to control powdery mildew.
Materials and methods

Cultivars Fiesta and 107 were used in a tunnel experiment in 1999-2000, and cultivars Nibla and Parker in a greenhouse experiment in 2000-2001. The experiments were carried out in Yair research station, located in southern Israel (Arava region), under organic management. Treatments (in four replications) included Neemgard (97% neem-oil), Sulfo-li (mineral sulfur (650 gr/l), Kaligrin (potassium bicarbonate), Rifol (70% fish oil), compost water extract (CEX), and AQ 10 (*Ampelomyces quisqualis*). Treatments were applied weekly, starting 50 days after planting, and disease assessment was done every two weeks, by evaluation of the infected leaf area (%), in a sample of 30 leaves from each treatment. Fruits were graded and weighed according their quality.

Results and discussion

Natural infections of powdery mildew on the control plants appeared 65 days post planting (dpp). Disease incidence in Nibla and Parker was 41 and 22%, respectively, 86 dpp, and 28 days later reached 100% (Figure 1). In all treatments, the maximal infection rate was 25% at the end of the season. Disease incidence was significantly reduced by Neemgard, Kaligrin, AQ10 and CEX treatments, as in the Sulfo-li treatment (Figure 1). Rifol treatment, applied only in cultivar Turkal, was also efficient, with 0.25% disease incidence at the end of the season (data not shown).

The yields were significantly lower (by 31-41%) in the control plants, as compared with all other treatments, only in cultivar Nibla, because of its relatively high susceptibility to the disease (Table 1). The yield obtained from the control plants of cultivar Parker did not differ
from that of the treated plants, indicating its relative resistance to the disease. The yields of grade A fruits (for export) in cultivar Nibla were 3.2, 3.1, 2.6, 2.5 and 2.5-folds higher than in the control using Neemgard, CEX, Sulfo-li, Kaligrin and AQ10, respectively, indicating that all the tested treatments may serve as an alternative to the common sulfur treatments in controlling powdery mildew on organic pepper. Previous reports demonstrated the efficiency of CEX in the control of grey mould (*Botrytis cinerea*) on tomato and pepper (Elad & Shtienberg, 1994) and early blight (*Alternaria solani*) on tomato (Tsror & Barak, 1998). The control of various diseases by foliar application of inorganic salts, such as potassium bicarbonate (Homma & Arimoto, 1990), bicarbonate solutions (Fallik et al., 1997), monopotassium phosphate (Reuveni et al., 1998) was also previously demonstrated. However, in the present study, the experiments were conducted on a large scale under field conditions and full organic management, demonstrating that the control of powdery mildew on peppers is possible using sulfur-alternative agents.

Table 1: The effect of foliar organic treatments on pepper yield levels

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Export market</th>
<th>Domestic market</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weight (kg/plot)</td>
<td>Number/plot</td>
</tr>
<tr>
<td>Nibla</td>
<td>Control</td>
<td>5.3 ± 1.3</td>
<td>35.0 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>Neemgard</td>
<td>16.9 ± 3.9</td>
<td>156.5 ± 38.4</td>
</tr>
<tr>
<td></td>
<td>Kaligrin</td>
<td>13.1 ± 2.3</td>
<td>81.3 ± 15.8</td>
</tr>
<tr>
<td></td>
<td>CEX</td>
<td>16.2 ± 4.0</td>
<td>93.8 ± 24.2</td>
</tr>
<tr>
<td></td>
<td>AQ10</td>
<td>13.0 ± 3.0</td>
<td>102.8 ± 23.2</td>
</tr>
<tr>
<td></td>
<td>Sulfo-li</td>
<td>13.7 ± 2.8</td>
<td>90.0 ± 21.6</td>
</tr>
<tr>
<td>Parker</td>
<td>Control</td>
<td>12.9 ± 2.2</td>
<td>88.5 ± 16.1</td>
</tr>
<tr>
<td></td>
<td>Neemgard</td>
<td>12.5 ± 2.0</td>
<td>82.5 ± 13.5</td>
</tr>
<tr>
<td></td>
<td>Kaligrin</td>
<td>14.9 ± 2.3</td>
<td>89.5 ± 14.7</td>
</tr>
<tr>
<td></td>
<td>CEX</td>
<td>14.9 ± 2.8</td>
<td>91.3 ± 18.8</td>
</tr>
<tr>
<td></td>
<td>AQ10</td>
<td>13.5 ± 2.6</td>
<td>80.5 ± 16.2</td>
</tr>
<tr>
<td></td>
<td>Sulfo-li</td>
<td>12.2 ± 1.9</td>
<td>87.0 ± 14.0</td>
</tr>
</tbody>
</table>

References


Post-harvest
We had previously shown that wound competence of postharvest biocontrol agents (BCAs) acting against wound pathogens of apples relies on resistance of these microorganisms to reactive oxygen species (ROS: superoxide anion, $O_2^-$ and hydrogen peroxide, $H_2O_2$), generated by the fruit tissue as a consequence of wounding. In this work we report the in vitro comparison of the enzyme activities deactivating ROS (superoxide dismutase, SOD and catalase, CAT) and the antioxidant potential of culture filtrates of two biocontrol yeast strains displaying lower (Rhodothorula glutinis LS11) and higher (Cryptococcus laurentii LS28) wound competence and antagonistic activity. The more efficient antagonist LS28 showed significantly higher SOD and CAT activities and higher antioxidant potential of its culture filtrate. Recently, the EU has released regulations (472/2002 and 1425/2003) setting the highest tolerable levels of the mycotoxins Patulin and Ochratoxin A (OTA), which contaminate apple-based food products and wine, as a consequence of infections by *Penicillium expansum* and *Aspergillus carbonarius* on apples and grape, respectively. Two biocontrol agents of our collection (strain LS11 and *Aureobasidium pullulans* LS30) are able to degrade in vitro Patulin and OTA, respectively. Further, strain LS11 shows active reduction of Patulin also in vivo, i.e. in apples artificially infected by *P. expansum*. 

Phenotypic traits underlying wound competence of postharvest biocontrol yeasts and degradation of mycotoxins by these microorganisms

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Evaluation of population density of *Pichia anomala* strain K and *Candida oleophila* strain O and their protection against *Penicillium expansum* Link on apples

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**Abstract:** The application of *Pichia anomala* (strain K) and *Candida oleophila* (strain O) in practical conditions had previously offered high protection against *Penicillium expansum* only when an antagonist density of $10^4$ cfu/cm$^2$ was detected on the intact surface of fruit. According to this, the initial concentration of application in controlled conditions for each antagonistic yeast was defined in order to obtain this level. Whatever the strain studied, the initial application concentration of $10^8$ cfu/ml allowed to obtain a density of yeast on the intact surface of apple higher than $10^4$ cfu/cm$^2$ after 24 hours, whereas a density lower than $10^5$ cfu/cm$^2$ was observed when an initial application concentration of $10^5$ cfu/ml on apple surface is used. Three scenarios reflecting the practical conditions of biological application were then tested with different periods of incubation between biological treatment, wounding of fruit surface and pathogen inoculation. Whatever the strain, the initial application concentration of $10^8$ cfu/ml allowed to obtain the highest densities of yeast's per wound and the highest protective levels in comparison to the use of initial application concentration of $10^5$ cfu/ml. The protective levels were positively correlated with the density of yeast determined in the wounded sites. Furthermore, these protective levels were influenced by wound wetness. The protective levels registered on wet wounds ranged between 52 and 100% while those observed on dry wounds did not exceed 30%. Whatever the scenario used the yeast density per wound and the protective levels induced by strain O were higher than those observed for strain K.

**Key words:** Biological control, *Candida oleophila* strain O, *Pichia anomala* strain K, apple, *P. expansum* Link, density of yeast by wound, protective levels

**Introduction**

*Pichia anomala* (strain K) and *Candida oleophila* (strain O) were previously selected for their high antagonistic activity (even after their mass production and drying) against *Botrytis cinerea*, *Penicillium expansum*, two wound pathogens causing economically important losses of Golden Delicious apples on storage rooms (Jijakli et al., 1999). The use of antagonistic yeasts in orchard conditions in order to control post-harvest diseases is still very limited, while their application just before harvest should allow the precolonisation of the fruit surface before wounding that occur during harvest and thus before the deposit of conidia of most wound pathogens (Ippolito & Nigro, 2000). Trials efficacy carried out under practical conditions with *P. anomala* strain K on apples showed that the high protective levels were always associated with a population density of strain K superior to $10^5$ cfu/cm$^2$ of surface fruit just after harvest (Jijakli et al., 2002).

In this context, our objectives consisted of 1) evaluating the antagonistic population density on fruit surface and 2) assessing the efficacy of both antagonistic yeasts in relation to their population densities in three scenarios reflecting practical conditions.
**Materials and methods**

**Measurement of antagonistic population density on intact fruit surface**

Apples fruits 'Golden delicious' were disinfected by soaking during two minutes in sodium hypochlorite solution (10%) then rinsed twice in sterile distilled water. After drying for one hour, fruits were treated by various concentrations of *C. oleophila* (strain O) or *Pichia anomala* (strain K) (10^5, 10^6, 10^7 and 10^8 cfu/ml) by dipping in a suspension of 350 ml during 2 minutes. Treated fruits were kept in ambient temperature. After 24 hours, the recovery of yeast on intact fruit surface was performed. Apples were introduced into plastic bags of 3000 ml. Each bag contained 4 apples and 1000 ml of washing KBPT buffer [6.8 g of KH2PO4 (0.05 M), 8.71 K2HPO4 (0.05 M) and 500 µl of Tween 80] (one plastic bag per treatment). Plastic bags were centrifuged during 20 minutes at 120 rounds/minute in order to yield the population of yeast strains from apple surface. After agitation, washing waters were diluted before plating onto Potato Dextrose Agar (PDA) medium. Three plates were used per dilution and per yeast concentration. The enumeration of cfu was carried out after 72 hours of incubation at 25°C. The mean surface of the apples was evaluated by means of as previously described (De Clercq et al., 2003) linear relationship between the surface of apples and their volume measured by water displacement [Surface (cm²) = 0.488 x volume displaced water (ml) + 66.1 with r = 0.99)]. Three trials were carried out over time and each treatment contained 3 replicates per trial.

**Efficacy assessment of both antagonistic yeasts in relation to their population densities in three scenarios reflecting practical conditions**

Ten-day-old colony of *P. expansum* grown on Potato Dextrose Agar (PDA) was used to obtain spore suspensions in sterile distilled water containing 0.05% of Tween 20 per litre. Spore suspensions were adjusted to 1 x 10^5 spores/ml using a Bürker cell. A volume of 10 µl of this suspension was used to infect wounded apple fruits.

Disinfected fruits were handled by soaking in a suspension of strain K or strain O at concentration of 10^5 or 10^8 cfu/ml during two minutes then wounded (4 wounds by fruit) at the equatorial site. Each wound was 2 to 3 mm in diameter and 4 mm in the deep. The biological treatment sequence of fruits was organized following three scenarios: 1) wounds were imposed directly after biological treatment. The recovery of yeast in wounded sites or the pathogenic inoculation were made 24 hours after wounding; 2) wounds were realized 24 hours after biological treatment. The recovery of yeast in wounded sites or the pathogenic inoculation were carried out immediately after wounding and 3) wounds were realized 24 hours after biological treatment. The recovery of yeast in wounded sites or the pathogenic inoculation were made 24 hours after wounding.

Whatever the scenario and the treatment combination strain-concentration studied, a set of 4 apples (4 wounds/ apple) was used to analyze the population density in wounds on fruits. The wounded sites were taken by means of a scalpel. Each site was separately placed in a solution of 10 ml of KBPT ‘washing’ buffer and crushed during 1 minute and 30 seconds by Ultra-thurrax T25. Hundred µl of each treatment wound were plated onto Petri dishes (3 replicates/wound site) containing PDA. Petri dishes were incubated at 25°C during 72 hours and yeast colonies of white colour were counted. The statistical analysis was made by ANOVA and Duncan’s test (P≤0.05) was used to distinguish the mean values.

A set of 20 apples (4 wounds per apple, 4 apples per treatment strain-concentration and 4 apples for the control) was also used for each yeast strain to measure the efficacy against *P. expansum*. After inoculation of the wounded sites, fruits were kept at 20°C during 7 to 11 days. A percentage of protection was calculated based on comparison of lesion diameter of
treated wounded sites and untreated wounded sites but inoculated. Four replicates were carried out per trial. The trial was not repeated.

**Results and discussion**

*Evaluation of population density of both antagonistic yeasts on apples surface fruits*

The recovery of yeast on fruit surface was made 24 hours after their application to estimate the population density of yeast per cm² of apple surface for each initial yeast concentration of application. The initial concentrations of $10^7$ and $10^8$ cfu/ml allowed obtaining a density of strain O superior to $10^4$ cfu/cm² of apple surface. Whereas for strain K, only the initial concentration of $10^8$ cfu/ml allowed to reach a similar population density.

![Graph](image_url)

**Figure 1.** Population density of both yeast strains K and O (cfu/cm²) on intact surface of apple fruit in relation to their initial concentrations of application (cfu/ml) after 24 hours. Points represent the means values calculated from 2 trials carried over time. Each trial contains 3 replicates per treatment. Bars represent the standard errors corresponding to their respective means.

*Evaluation of population density of yeast strains K and O and their efficacy in three scenarios reflecting practical conditions*

Two initial concentrations, $10^8$ cfu/ml (allowing having a yeast density higher than $10^4$ cfu/cm² on the intact surface of apple fruit) and $10^5$ cfu/ml (allowing having a yeast density lower than $10^4$ cfu/cm² on intact surface of apple surface fruit) were used in these experiments.

**Scenario I**

In the case of wounds made directly after the biocontrol treatment, the recovery of each antagonistic strain in wounded apples showed that the highest levels of populations were detected for strain O at both concentrations of application in comparison with strain K (Fig.2). The highest protective level was attributed to the initial concentration of application of $10^8$ cfu/ml whatever the yeast strain. Strain O offered 83.9% and 100% of protection respectively for treatments at $10^5$ and $10^8$ cfu/ml, while the protective levels due to strain K were 52.5% and 71.1% respectively for the same treatments (Figure 2).
Figure 2. Population densities of two yeast strains and their efficacy against *P. expansum* (10^5 conidia/ml) in wounded sites realized directly after biological treatment of apples by soaking in two initial concentrations of application 10^5 and 10^8 cfu/ml. The recovery of yeast in wounded sites or the pathogen inoculation were made 24 hours after wounding.

Figure 3. Population densities of two yeast strains and their efficacy against *P. expansum* (10^5 spores/ml) on wounded sites realized 24 hours after biocontrol treatment. The recovery of yeast and the pathogenic inoculation were made immediately after wounding.

**Scenario II**
The recovery of both strains in wounded sites (applied 24 hours before the creation of wounds) showed the total absence of colonies at treatment of 10^5 cfu/ml (Figure 3). While at treatment 10^8 cfu/ml, the average number of colony by wound was significantly higher and varies between 90 and 120 cfu/wound for both yeast. Whatever the initial concentration of application of each antagonistic strain, the efficacy reported against *P. expansum* did not exceed 30%.

**Scenario III**
In this scenario, population densities in wounded fruits for both initial concentrations of application were higher with strain O than with strain K. Whatever the strain, an initial concentration of application of 10^8 cfu/ml gave an average number of colonies significantly higher than the number obtained with treatment at 10^5 cfu/ml (Figure 4). The protective levels offered by strain O (C. oleophila) against the blue decay were 72.2% and 59.5% respectively for treatments at 10^8 and 10^5 cfu/ml. For strain K, these levels reached 62.1% and 42.8% respectively for both treatments. The higher protective levels observed in scenario I and III were associated with a density of yeast on apple exceeding 10^4 cfu/cm² of intact apple surface due to an initial concentration of application of 10^8 cfu/ml whatever the antagonistic yeast strain. This was not the case in scenario II where population densities at the wound site were very low whatever the initial concentration of yeast application.
The scenario III is reflecting the most frequent practical conditions in case of pre-harvest application of yeast one or two days before harvesting and handling. These manipulations created wounds before storage. The protective levels were positively correlated with the population densities whatever the scenario and the yeast [Strain O: 11,20X + 4,77 (r = 0,93) and strain K: 17, 13X – 0,21 (r = 0,96)].

Mercier & Wilson (1995) studied the effect of the humidity on the growth of C. oleophila and the protective level against B. cinerea on apples. The population of this strain increased quickly when water was periodically applied at the wounded sites of the fruit. These authors supposed that the humidity could be a factor limiting the development of antagonistic microorganisms. Our scenarios reflected also different situations of humidity at the wound site. The protective levels registered on wet wounds ranged between 52 and 100% (scenario I) while those observed on dry wounds did not exceed 30% (scenario II). Our results suggest that the humidity controlled the population densities of yeasts and their protective levels.

Figure 4. Population densities of both yeast strains and their efficacy against P. expansum (10^5 conidia/ml) on wounded sites realized 24 hours after biological treatment. The recovery and pathogenic inoculation were realized 24 hours after wounding.

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References


Comparative study of chitinase and pyrrolnitrin biocontrol activity in *Serratia plymuthica* strain IC1270

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**Abstract:** Rhizosperic strain of *Serratia plymuthica* IC1270 shows wide-range antagonism toward plant-pathogenic fungi and produces several antifungal compounds, including the antibiotic pyrrolnitrin [Prn, (3-chloro-4-(2’-nitro-3’-chlorophenyl)-pyrrole)] and a set of chitinolytic enzymes, consisting of two N-acetyl-β-D-glucosaminidases and an endochitinase ChiA. Under greenhouse conditions, the bacterium reduced the incidence of several fungal diseases, *Rhizoctonia solani* root rot in cotton and bean, and *Pythium aphanidermatum* damping-off in cucumber among others. Since both the chitinolytic and antibiotic compounds were shown to be involved in the microbial antagonism, the main goal of this work was to specify the relative input of Prn and ChiA in strain IC1270's biocontrol activity. To clarify the relative input of these compounds in the bacterium biocontrol activity two mutants were obtained by gene-replacement technique. One of the mutants, IC1270#C7, deficient in chitinolytic activity and another one, IC1270#P1, deficient in Prn production, were compared against *R. solani* and *P. aphanidermatum* in vitro and in greenhouse. Whereas mutant C7 hardly differed from the parental strain, P1 mutant lost its antifungal activity almost completely. The results demonstrate prevalent role of Prn for strain IC1270 biocontrol activity against these fungal pathogens.

**Keywords:** chitinases, pyrrolnitrin, bacterial antagonists, soil-borne fungal diseases, biocontrol

**Introduction**

Most biocontrol strains of bacteria are known to produce one or more of a diverse array of potent antifungal metabolites. Various species of soil bacteria described as antagonists of phytopathogenic bacteria and fungi produce secondary metabolites with strong antibiotic activity. The best characterized are simple compounds such as phenazines, 2,4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin. Pyrrolnitrin (3-chloro-4-[2’-nitro-3’-chlorophenyl] pyrrole) (Prn) producing strains and the purified antibiotic are effective *in vitro* as antagonists of many fungi, bacteria and yeast. The Prn biosynthetic locus from *P. fluorescens* strain BL915 contains four genes (*prnABCD*) and is highly conserved among Prn-producing pseudomonads (Hammer et al., 1999).

Biological control of chitin-containing plant pathogens has been correlated with chitinolytic enzyme production. Therefore, many chitinolytic soil and rhizosphere bacteria and fungi are potential BCA (Chernin and Chet, 2002). Strain IC1270, isolated from rhizosphere of grape, was previously described as *Pantoea agglomerans* and recently re-identified as *Serratia plymuthica* on the basis of 16S rRNA partial sequence analysis (GeneBank accession no. AY551332). The strain was shown a wide host range antagonists of
a number of fungal and bacterial plant pathogens in vitro and under greenhouse conditions. It produces a set of secreted chitinolytic enzymes consisting of a 58-kDa endochitinase (EC 3.2.1.14) ChiA, and two N-acetyl-β-1,4-D-glucosaminidases (EC 3.2.1.30) of 89 and 67 kDa (Chernin et al., 1995), Prn (Chernin et al., 1996), still non-identified siderophore(s) and an exoprotease. Although both chitinases and Prn have known essential for biocontrol capacity of many bacterial BCAs, our goal was to estimate the relative input of these compounds in strain IC1270 antifungal activity. Two mutants, IC1270#C7 (chiA⁻), deficient in chitinolytic activity and IC1270#P1 (prnC⁻) deficient in Prn production, were obtained by gene-replacement technique and their biocontrol activity was compared to that of the parental strain in greenhouse against *Rhizoctonia solani* on bean and *Pythium aphanidermatum* on cucumber. Whereas mutant C7 hardly differed from the parental strain in ability to suppress *R. solani*, P1 mutant lost its antifungal activity against *R. solani* almost completely and only residual level of biocontrol was observed against *P. aphanidermatum*. The results demonstrate prevalent role of Prn for strain IC1270 antifungal activity.

**Materials and methods**

**Chitinolytic activity assay**

Cells were seeded on plates with semi-minimal medium supplemented with colloidal chitin (0.2% w/v) and solidified with 1.5% agar. The plates were incubated at 28°C for 96 h until zones of clearing of the chitin could be seen around the colonies.

**Preparation of extracellular proteins and detection of chitinolytic enzymes**

The procedure was generally performed as described previously (Chernin et al., 1995). Liquid or solid synthetic medium with 0.2% (w/v) colloidal chitin as the sole carbon source was used to induce chitinolytic activity of the bacteria. Secreted proteins denaturated with sodium dodecyl sulfate (SDS) were separated by 10% polyacrylamide gel electrophoresis (PAGE) and then reactivated by removing SDS using the casein-EDTA procedure. Enzyme activity was detected on gels by using fluorescent 4-methylumbelliferyl analogues of dimeric, trimeric and tetrameric chitin as substrates.

**Isolation of Prn**

The procedure was generally performed as described previously (Chernin et al., 1996). The crude extracts (50 µl) were applied to HPLC system using the reverse-phase C18 column. Prn absorption was monitored at 225 nm. HPLC-purified Prn preparations were dissolved in methanol and screened for antibiotic activity towards the tested fungi in bioassay on plates.

**Gene-replacement mutagenesis**

The genes subjected to mutagenesis were: i) a homologue of the *prnC* gene of *Pseudomonas fluorescens*, known as one of four genes required for the synthesis of Prn in this bacterium, cloned and sequenced from strain IC1270, and ii) the strain IC1270 gene *chiA* encoding the 58-kDa endochitinase (Chernin et al., 1997). The *chiA* and *prnC* genes cloned into pGEM-T Easy vector were inactivated by insertion of a gentamicin (Gm³) cassette; the interrupted genes were then cloned into a pEX18Tc gene-replacement vector, transferred into strain IC1270 and the double-crossover progeny were selected as described (Hoang et al., 1998).

**Inhibition of *R. solani* and *P. aphanidermatum* under greenhouse conditions.**

*R. solani* infection of beans (*Faseolus vulgaris* L., cv. “Wax yellow”) and *P. aphanidermatum* infection of cucumber (*Cucumis sativus* L. cv. “Cfr 413”) were used as the model systems. Briefly, in the case of infection with *R. solani*, polypropylene boxes were two-thirds filled with sandy loam soil (pH 7.2). A seed cover layer (one-third of the pot's depth) was infected with a preparation of *R. solani* mycelium blended and mixed with soil. Bacteria in tap water
(ca. $10^9$ cells/ml) were applied by drench immediately after infection and on the fourth day from sowing. In the case of *P. aphanidermatum* infection the soil was inoculated by oospore suspension (~$5 \times 10^5$ spores/ml). Before sowing, the seeds were soaked for 10 min in the bacterial suspension (~$10^9$ cells/ml in tap water) being tested, then the seeds were sowed in the soil and additionally inoculated by dripping 100 µl of the same suspension of bacterial cells onto the seeds before covering them with the infected soil. In both schemes of experiments 10 seeds were placed in each box. In the disease control, tap water was used instead of the bacterial suspension. In the seed germination control, the soil was not infected with fungal pathogen and the pots were irrigated only with tap water. Disease incidence was determined after 10 to 14 days as the percentage of seedlings with *R. solani* root rot or *P. aphanidermatum* pre- and post-emergence damping-off diseases.

**Results and discussion**

*Isolation of gene-replacement mutant*

The genes subjected to mutagenesis were: i) strain IC1270's gene *chiA* encoding the 58 kDa-endochitinase and ii) strain IC1270's homologue of *Pseudomonas fluorescens* gene *prnC* required for Prn synthesis. The *chiA* and *prnC* genes of IC1270 were cloned into pGEM-T Easy vector and inactivated by insertion of gentamicin cassette, the interrupted genes were then cloned into a pEX18Tc gene replacement vector, transferred into strain IC1270 and the double-crossover progeny were selected using the *sacB*-based strategy (Hoang et al., 1998). The gene-replacement events were then further verified by PCR and Southern analysis (data not shown). Two mutants, one (IC1270#C7) with replacement in gene *chiA* and another one (IC1270#P1) with replacement in gene *prnC* were selected for further analysis.

*Characterization of IC1270#C7 and IC1270#P1 mutants.*

Antifungal activity in vitro. Strains IC1270 and IC1270#C7, but not strain IC1270#P1, suppressed growth of *R. solani* and *P. aphanidermatum* on plates.

Chitinolytic activity of IC1270 and its mutants. Strains IC1270 and IC1270#P1, but not strain IC1270#C7, hydrolyzed colloidal chitin after 72 to 96 h of growth on solid minimal medium supplemented with colloidal chitin as the sole carbon source.

Identification of chitinolytic enzymes. Wild-type strain IC1270 and its Prn-deficient mutant IC1270#P1, but not the IC1270#C7 (*chiA*) mutant, produced and excreted three chitinolytic enzymes: the 89-kDa and 67-kDa-exocitinases, and the 58-kDa endochitinase ChiA. A deficiency in the production of all three enzymes by the IC1270#C7 mutant due to gene-replacement in *chiA* suggests that the corresponding structural genes are organized into one transcriptional unit and that the mutation in *chiA* has a polar effect.

Prn production. HPLC and bioassays verified mutant IC1270#P1's total deficiency of in Prn production, while the IC1270#C7 mutant produced Prn to the same level, in average, as the parental strain.

Greenhouse experiments. Biocontrol activity of strain IC1270 and its mutants was tested under greenhouse condition using the basidiomycete *R. solani*, which contains mainly β-glucan and chitin, but no cellulose in the cell-wall, and the oomycete *P. aphanidermatum*, whose cell-wall is composed mainly of β-glucan and cellulose, as model plant pathogens. Whereas IC1270#C7 hardly differed from the parental strain, the antifungal activity of IC1270#P1 drastically decreased (Table 1), indicating the predominant role of Prn in controlling both of these fungi.
Table 1. Biocontrol activity (IDR,%)*

<table>
<thead>
<tr>
<th>Strain/fungus</th>
<th><em>Rhizoctonia solani</em></th>
<th><em>Pythium aphanidermatum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>IC1270</td>
<td>48.4±4.6a **</td>
<td>78.4±8.8a</td>
</tr>
<tr>
<td>IC1270#P1</td>
<td>4.3±3.2b</td>
<td>26.2±4.6b</td>
</tr>
<tr>
<td>IC1270#C7</td>
<td>46.1±4.9a</td>
<td>64.2±4.9a</td>
</tr>
</tbody>
</table>

* Biocontrol activity against *R. solani* root-rot disease in bean and *P. aphanidermatum* damping-off disease in cucumber is presented as percentage of the index of diseases reduction (IDR) estimated for each of the fungal treatment. Disease level was calculated relative to 100% disease control (the actual average level of disease was 66.8 ± 4.9 for *R. solani* and 86.8 ± 8.9 for *P. aphanidermatum*). Three to four independent experiments were performed with four to six repetitions per treatment.

** Different letters in the same column indicate significant differences between means using the all pairs Tukey-Kramer test (*P*≤0.05). Homogeneity of the variances between repetitions in all experiments was proven with a Bartlett test (*P*≤0.05).

The data show that the biocontrol ability of *S. plymuthica* strain IC1270, which produces a set of chitinolytic enzymes and antibiotic pyrrolnitrin, against the plant-pathogenic fungus *R. solani*, relies mainly on the production of the antibiotic; a deficiency in the production of the chitinolytic enzymes does not cause significant reduction in biocontrol. Prn was also shown to be responsible for control of *P. aphanidermatum* by strain IC1270. However, the observed residual activity of the Prn- mutant IC1270#P1 against this fungus indicates that other mechanisms (e.g. competition for nutrients and induced resistance) may be involved in the biocontrol provided by strain IC1270 as well.

References


Formulation and shelf-life studies of the biocontrol yeast

**Pichia anomala**: positive effect of endogenous solutes, isotonic solutions and additives during fluidised-bed drying

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**Abstract:** The major hurdle in production of commercial biocontrol agents (BCAs) has been the lack of production of formulations of the right quality and shelf-life. Of particular importance is the conservation of viability and ecological competence after application. With this in mind studies were conducted to develop formulations of *Pichia anomala* which would have these attributes. Mokiou & Magan (2002) showed that by manipulation of the physiology of *P. anomala* cells during growth it was possible to modify the endogenous trehalose content of the cells. When cells were suspended in isotonic solutions based on NaCl, a solute with no adverse effect on yeast cell viability, the amount of trehalose detected was much higher. Studies with fluidised bed-drying examined several additives for conservation of viability and showed that cotton seed flour+skimmed milk was best treatment. During a five month-storage period at 4°C, formulated yeast cells with a high trehalose intracellular concentration, retained high viability (up to 54%). Moreover, use of isotonic solutions resulted in improved shelf-life of formulated yeast cells; cells suspended in water had 42% viability while those in NaCl isotonic solutions retained 52% viability for the same storage period. These treatments were effective at controlling *Penicillium* and other spoilage fungi in moist grain.

**Key words:** *P. anomala*, trehalose, fluidised bed-drying, isotonic solutions, formulation, shelf-life

**Introduction**

Usage of fungal biological control agents (BCAs) as an alternative method to chemicals appears favourable since public concern about health and environmental matters are combined with the ban or withdrawal of several pesticides. However, BCAs are living organisms and their economic production process, formulation, distribution and application are of great importance and require special considerations. The major obstacle in the commercialisation of BCAs products is the development of a shelf-life-stable formulated product that retain efficacy similar to those of fresh BCA cells (Janisiewicz & Jeffers, 1997).

Reducing the metabolic rate of microorganisms by removing the available water has been the principle method used to preserve cultures. Drying microorganisms enables preservation of the inoculum over a long period of time, maintaining high viability, and does not require cool temperatures during storage and distribution. Therefore, normal storage and distribution channels can be used making the final product reasonably cheap (Rhodes, 1993).

Fluidised bed-drying has been extensively used to manufacture active dry yeast on a large scale (Bayrock & Ingedew, 1997; Larena et al., 2003) dried *E. nigrum* conidia by using different drying methods and fluidised bed-drying retained 100% viability of conidia.

Björnberg & Schnürer (1993) first showed that *Pichia anomala* J121 strain effectively reduced growth of *Pichia roqueforti* and *Aspergillus candidus in vitro. *P. anomala* has also been shown to reduce ochratoxin A accumulation in co-culture with *P. verrucosum*. 
Hallsworth & Magan (1995) showed that elevated concentrations of the disaccharide trehalose, due to response to osmotic stress, in conidia of entomopathogenic fungi prolonged shelf-life. Mokiou & Magan (2002) showed that *P. anomala* cells intracellularly accumulated trehalose when exposed to water stress by addition of proline and NaCl to molasses media. The objective of this study was to investigate the perspective of effectively formulating *P. anomala* by use of fluidised bed-drier and the impact that endogenous solutes, isotonic solutions and additives might have to viability and shelf-life of the formulated yeast cells.

**Materials and methods**

*Microorganism, culture media used and their modification*

The microorganism used in this study was *Pichia anomala* (strain J121). Cane molasses-based medium made of cane molasses 40g/l and urea 1.2g/l. Modification of media aw was previously described by Mokiou & Magan (2002).

*Extraction and quantification of intracellular polyols and sugars*

Extraction of polyols and sugars was done according to Mokiou & Magan (2002).

*Fluidised bed-drying*

*P. anomala* cells produced were washed twice in HPLC grade water or in NaCl 0.98 aw isotonic solution and centrifuged. Carriers were added at a proportion 1:1 (w/w, *P. anomala* cells/carrier) and adjuvants at 10% (w/w); the mixtures resulting were introduced in fluidised bed-dryer 350s (Burkard Manufacturing Co. Ltd, Hertfordshire, UK) and dried at 50°C for 20 mins. Viability, by using viablue stain (Hutchenson et al., 1998) for yeast was then measured.

*Storage trials*

Formulated *P. anomala* cells were kept in plastic tubes at ambient and fridge (4°C) temperature. Viability was checked overtime by resuspending cells in distilled water+Tween 20+Agar for 10 mins at 30°C. Viablue stain was then added (Hutchenson et al., 1998).

**Results**

*Trehalose intracellular accumulation in cells washed with water and isotonic solutions*

The changes obtained in trehalose content of *P. anomala* cells grown in control, proline and NaCl in 0.98 aw molasses media when cells were washed with water (A) and isotonic solutions (B) are shown in Figures 1 and 2. In the unmodified media trehalose intracellularly accumulated at 34.9 mg/g yeast f.w. In proline 0.98 aw treatment, trehalose was found to be strongly intracellularly accumulated (61.24 mg/g yeast f.w.). However, when cells were washed with isotonic solution the amount of accumulated trehalose was much higher (89.7 mg/g yeast f.w.). In NaCl 0.98 aw treatment, trehalose accumulated to 29.4 mg/g yeast f.w (A) and when the same cells were washed with isotonic solution (B) trehalose accumulated to 113.4 mg/g yeast f.w, almost 4 times more.

*Fluidised bed-dried formulated P. anomala cells storage trials*

Figure 3 shows the temporal changes of formulated *P. anomala* cells viability over a period of 5 months storage. Cells were grown in control, proline 0.98 aw and NaCl 0.98 aw molasses media and fluidised bed-dried as formulation of Cottonseed Flour: 1+10%SM for 20 mins at 50°C. Cells were washed with water and NaCl isotonic solution prior to drying and stored at 4°C. Control cells initially had 73% viability and after 5 months storage decreased to 54%. Proline 0.98 aw cells, when washed with water, had initial viability of 71% which decreased to 53% at the end of the storage trial. Those washed with isotonic solutions had 67.5% viability which decreased to 54%. NaCl 0.98 aw cells when washed with water had an initial 67.5%
viability decreased to 42% after 5 months storage. NaCl 0.98 a\textsubscript{w} cells, when washed with isotonic solution had an initial 73% viability which ended at 52% viability after storage.

![Graph A](image_url)

![Graph B](image_url)

Figure 1. Endogenous concentration of compatible solutes of *P. anomala* cells grown in Proline modified molasses media after 72 h incubation at 25°C. Cells were washed with water (A) and isotonic solution (B) prior to extraction.

**Discussion**

This study is the first investigation of effectively formulating *P. anomala* cells using a fluidised bed-drier and the impact that endogenous solutes, isotonic solutions and additives have on the viability/shelf-life of the formulated cells. It has been shown that the amounts of trehalose accumulated and, more importantly, retained intracellularly play a key role in the drying and storage process. *P. anomala* cells, grown in NaCl and proline 0.98 a\textsubscript{w} modified molasses media, retained much higher trehalose amounts when washed with isotonic solutions. In the case of NaCl, the amount of trehalose detected when an isotonic solution was used was almost four times greater. Pascual et al. (1999) also found that trehalose and compatible solutes were washed out of *E. nigrum* conidia when separated from solid media and detected significant amounts in the washing liquids.
To formulate *P. anomala*, by fluidised bed-drying, several different additives were used with cotton seed flour + skimmed milk being the best of all in terms of viability. Of the different treatments used for a storage period of five months, *P. anomala* cells grown in proline-based molasses media were found to have decreased viability from 70% to 54%. Use of an isotonic solution did not modify this effect. However, in *P. anomala* cells grown in NaCl modified medium there was a significant increase in viability over a 5-month storage period when isotonic solutions were used. Big differences in trehalose intracellular accumulation, when isotonic solution was used, possibly accounted for this improved shelf-life. Bayrock & Ingledew (1997) showed that trehalose addition to compressed yeasts before drying resulted in 30% higher viability. Previous studies by Hallsworth & Magan (1994) pointed out that conidia with elevated trehalose concentrations remained viable for longer. This study has shown that formulating *P. anomala* cells using fluidised bed-drying should be an important consideration and that the use of additives and isotonic solutions, aiming at retaining trehalose in the cells, has a positive effect on final product shelf-life.

![Graph A](image1)

**A**

![Graph B](image2)

**B**

Figure 2. Endogenous concentration of compatible solutes of *P. anomala* cells grown in NaCl modified molasses media after 72 h incubation at 25°C. Cells were washed with water (A) and isotonic solution (B) prior to extraction.
Figure 3. Viability overtime of *P. anomala* cells grown in control, proline 0.98 aw and NaCl 0.98 aw molasses media and dried as formulation of cottonseed flour: 1+10%SM for 20 mins at 50°C. Cells were washed with water and NaCl isotonic solution prior to drying. Cells were stored in the fridge 4°C

**Acknowledgements**

This work was part of the European Union project QoL-PL1999-1065 (Biopostharvest). We are grateful to Prof. J. Schnürer for the *P. anomala* J121 strain. Stella Mokiou is grateful to the Konstantinos Lazaridis and Alexander Onassis foundations for funding.

**References**


Post-harvest biological control of a wide range of fruit types and pathogens by \textit{Pantoea agglomerans} EPS125

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Abstract: \textit{Pantoea agglomerans} strain EPS125 efficiently inhibited infections by \textit{Penicillium expansum} in apple and pear fruits either in commercial cold storage conditions under controlled atmosphere or in simulated market conditions. The strain also controlled \textit{Monilinia laxa} and \textit{Rhizopus stolonifer} in stone fruits (plum, peach, cherry, apricot and nectarine) and \textit{Botrytis cinerea} in strawberry fruits. Dose-response experiments of the effect of EPS125 and pathogen concentrations indicated that at medium to low pathogen dose, optimal EPS125 concentrations were above $10^7$ cfu/ml. The strain EPS125 colonizes, grows and survives well on wounds and less in non-wounded peel surface of pome and stone fruits. Significant inhibition of conidial germination and hyphal growth of \textit{P. expansum}, \textit{R. stolonifer} and \textit{M. laxa} was achieved when the fungal and EPS125 cells were co-cultivated on peel leachate, nectarine juice or apple juice. However, no effect was observed when the antagonist and the pathogen cells were physically separated by a membrane filter which permits nutrient and metabolite interchange. Therefore, a direct interaction between the strain and the pathogen cells is necessary for antagonism, without a significant contribution of the production of antibiotic substances or nutrient competition. Preemptive exclusion by wound colonization and direct interaction with the pathogen is proposed as the mechanism of biocontrol.

Introduction

Post-harvest rot of fresh fruits due to fungal infections causes significant economic losses to the fruit industry during storage, transport and in the marketing. The most important post-harvest diseases of fruits are caused by fungus like \textit{Penicillium} spp., \textit{Botrytis cinerea}, \textit{Monilinia laxa} and \textit{Rhizopus stolonifer} (Ogawa et al., 1995). Post-harvest pathogens are quite efficiently controlled by a combination of storage technologies, physical methods and synthetic chemical fungicides (Eckert & Ogawa, 1988; Conway et al., 1999). However, the limitation of the number of authorized active ingredients, the growing concern of consumers about chemical residues on fruits and the increasing resistance of some fungal pathogens to commonly used fungicides have increased efforts to develop alternative or complementary methods like biological control (El-Ghaouth & Wilson, 1995; Janisiewicz, 1998). Biological control of post-harvest pathogens has been successful because several fungi and bacteria were reported to effectively reduce post-harvest diseases of peach, nectarine, apple, pear, citrus, cherry, kiwi and grape. Several microbial fungicides for post-harvest rot control are currently under varying degrees of development and some yeasts and bacteria are already available in the market (Fravel et al., 1999). However, in most cases the microbial fungicides have a relatively narrow host and fruit type spectrum of activity in comparison with the wide range of many synthetic fungicides (Janisiewicz & Bors, 1995).

In a screening program performed in our laboratory to isolate naturally occurring bacteria from plants, with potential application for biocontrol (Montesinos et al., 1996), a strain of \textit{P. agglomerans} named EPS125 was selected. The strain inhibits infections by \textit{R.}
*stolonifer* and *M. laxa* on peach, apricot and nectarine (Bonaterra et al., 2003). The present study was conducted to determine: 1) the range of activity using four fungal pathogens and eight fruit species of different cultivars; 2) the ability of the antagonist to colonize, survive and growth in wounds and on the surface of fruits and 3) the putative mechanism of action.

**Materials and methods**

The strain EPS125 of *Pantoea agglomerans* was isolated using a selective enrichment technique from a surface of a Doyenne du Comice pear and is deposited in the Spanish Type Culture Collection with the referential code CECT 5392. The strain is the object of a patent with the reference number EP1 402779A1 and can be specifically identified by means of DNA fingerprinting using MRFILP patterns resolved by Pulsed Field Gel Electrophoresis (Montesinos et al., 2001). Strain EPS125 can be produced at semi-industrial scale by liquid fermentation and freeze drying. The strain was characterized phenotypically and genotypically, and according to acute toxicological studies does not produce primary dermal or eye irritation on rabbit, and the acute oral toxicity on rats was higher than $10^{10}$ cfu/Kg. A spontaneous mutant resistant to $100\ \mu\text{g ml}^{-1}$ of rifampicin, which retains the phenotypical and genotypical characteristics and performance of the parental strain, was used in the present study. The recovering of viable colonies of EPS125Rif in LB amended with rifampicin was 100% of the LB non-amended medium. For strain preservation cultures were grown in LB agar and stored in 20% glycerol at -80ºC.

The efficacy of EPS125 in the biological control of different pathogens responsible of postharvest rot of fresh fruits was tested in different kinds of fruits. In *P. expansum* assays, Williams, Conference and Kaiser pear cultivars, and Golden and Red Chief apple cultivars were used. In *R. stolonifer* assays the peach cultivar Red Haven and the plum cultivars Shiro and Sorriso di primavera were used. In *M. laxa* assays Van and Nero I cherry cultivars, Canino and Reale d’Imola apricot cultivars, and Venus and K-2 nectarine cultivars were used. In *B. cinerea* assays, the strawberry cultivar Idea was used. The fruits were treated either with a $10^8$ cfu/ml suspension of *P. agglomerans* EPS125 or with water (non-treated control). All the treatments were applied by immersion of fruits for 1 min into the treatment suspensions. Within the next two hours the fruits were inoculated by immersion for 1 min in a spore suspension of either *P. expansum*, *B. cinerea*, *M. laxa* or *R. stolonifer* at a concentration of approximately 1x10^4 spores/ml. Stone fruits (plum, peach, nectarine and apricot) were maintained at 15-20ºC and the incidence of infected wounds (%) was assessed after 3-7 days of incubation. Pears and apples were stored for 6 months either in standard cold storage (at -1ºC for pears and 0ºC for apples) or in controlled atmosphere cold storage (at -1ºC, 3% O_2 and 2% CO_2 for pears; and 0ºC, 2% O_2 and 2% CO_2 for apples). Pear cultivar Williams was maintained at 20 ºC for a week. After 6 months under cold storage, the fruits were maintained 7 days at 20ºC and the incidence of infected fruits was assessed.

Dose-response experiments were used to estimate efficiency parameters for the bio-control agent and pathogens using a hyperbolic saturation model (Montesinos & Bonaterra, 1996). The effect of pathogens and biological control agent concentrations on the severity of fruit rot was assessed at several concentrations of conidia of *P. expansum*, *M. laxa* and *R. stolonifer* (5x10^2, 1x10^3, 1x10^4, 5x10^4 conidia/ml) and of *P. agglomerans* EPS125 (0, 10^5, 10^6, 10^7, 10^8, 10^9 cfu/ml).

The ability of EPS125 to survive and multiply in wounds and on the surface of several fruits was studied at several cell concentrations (10^5, 10^7, 10^8 and 10^9 cfu/ml). The fruits were periodically sampled and the population levels (cfu/cm^3) were counted on LB agar plates supplemented with 100 µg/ml of rifampicin.
The effect of EPS125 cells on spore germination and mycelial growth of *M. laxa*, *P. expansum*, *R. stolonifer* was determined on peel leachate, nectarine juice or apple juice. We studied the effect of EPS125 cells on spore germination when antagonist cells and pathogen were in direct interaction and when they were physically separated using cylinder inserts provided with a membrane filter of 0.45 µm pore size which permits nutrient and metabolite interchange but not a direct contact between them.

**Results and discussion**

*P. agglomerans* EPS125 inhibited fungal rot (blue mold, gray mold, brown rot and soft rot) in pome (apple and pear), stone (cherry, plum, apricot, nectarine and peach), and strawberry fruits of different cultivars. The treatment of wounded fruits with EPS125 reduced decay caused by the pathogens significantly in 17 of the 20 trials performed, but the levels of efficacy varied depending on cultivars, pathogens and storage conditions. In brown rot (*M. laxa*) control trials the efficacy of EPS125 was high (78.5-96.1%) in 4 of the 6 trials performed on nectarine, apricot and cherry cultivars, and was moderate (32.5-40.7%) in two of the trials performed on apricot and cherry cultivars. In soft rot (*R. stolonifer*) control trials on plum and peach cultivars the efficacy was high (64.8-92.3%) in the three experiments performed. In blue mold (*P. expansum*) control trials on apple and pear cultivars the efficacy was high (62.5-97.0%) in 6, moderate (between 25.7 and 37.4%) in 2, and low (4.6-15.3%) in two of the ten trials performed. The efficacy of EPS125 in the control of gray mold (*B. cinerea*) of strawberry was moderate (55%). The lowest control was obtained on pears stored under controlled atmosphere. The efficacy of biocontrol depended on the concentration of the biocontrol agent and pathogen. At medium to low pathogen dose, optimal EPS125 concentrations were above $10^7$ cfu/ml.

Strain EPS125 colonized and survived better in wounds than on the surface of non-wounded apple, pear and plum fruits. In all the experiments performed the population levels in wounds were significantly different from the population levels on the non-wounded peel surface. Within the first hours after the treatment, population levels of *P. agglomerans* EPS125 in wounds ranged from 5.8 to 7.5 log$_{10}$cfu/cm. On the non-wounded fruit surface the population levels were highly variable depending on the fruit type and from trial to trial and ranged between 1.3 to 5.9 log$_{10}$cfu/cm. At the end of the storage period of 5 months under cold storage conditions in pear and apple fruits and during the 7 days of storage at 15 ºC in plum fruits, the population levels in wounds did not changed significantly in relation to the starting levels. However, upon additional incubations at 20 ºC once storage was resumed, the population levels were stable or in some cases slightly decreased. After this time, the wounds appeared healed without symptoms of necrosis or rot. Contrarily, the population levels on the non-wounded surface of fruits decreased with time upon storage and became not detectable in most cases at the end of the experiments.

Significant inhibition of conidial germination and hyphal growth of *P. expansum*, *R. stolonifer* and *M. laxa* was achieved when the fungal and EPS125 cells were co-cultivated on peel leachate, nectarine juice or apple juice. However, no effect was observed when the antagonist and the pathogen cells were physically separated by a membrane filter which permits nutrient and metabolite interchange. Therefore, a direct interaction between the strain and the pathogen cells is necessary for antagonism, without a significant contribution of the production of antibiotic substances or nutrient competition. Preemptive exclusion by wound colonization and direct interaction with the pathogen is proposed as the mechanism of biocontrol. Nowadays, transposon mutagenesis is being used to demonstrate the mechanisms responsible of the EPS125 biocontrol. A total of 4000 mutants have been obtained by
conjigation of EPS125 and *E. coli* λ*pir* strain (pCAM140) which contain a minitransposon with a promotorless reporter gene GUS and are now being proven by *in vitro* and *ex-vivo* assays.

**Acknowledgements**

This work was supported in part by the Spanish Government (Comisión Interministerial de Ciencia y Tecnología of Spain CICYT, Projects AGF98-0402 and PETRI) and Catalanian Government (Comissió Interdepartamental de Ciència I Tecnologia de Catalunya CeRTA, GRQ). We thank Cooperativa Girona Fruits, Cooperativa Costa Brava, and Mas Badia Agricultural Experiment Station for technical support.

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Mode of action of biocontrol agents
Systemic resistance in \textit{Arabidopsis thaliana} induced by biocontrol agent \textit{Trichoderma harzianum}

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\textbf{Abstract:} In Arabidopsis, earlier work with strains of non-pathogenic rhizobacteria demonstrated jasmonic acid- and ethylene-dependent induced systemic resistance (ISR) that is effective against different pathogens. In the present work, the mutant analyses showed similarity of the rhizobacteria- and \textit{Trichoderma}-ISR signaling pathways in which components from the jasmonic acid and ethylene response are engaged to trigger a defense reaction. However, not only jasmonic acid and ethylene but also other phytohormones such as abscisic acid, auxin and gibberellic acid are involved in activating resistance mechanisms in Arabidopsis against \textit{Botrytis cinerea}.

\textbf{Keywords:} \textit{Arabidopsis}, mutants, \textit{Botrytis}, TRICHODEX, ABA, auxin, gibberellin, ethylene

\textbf{Introduction}

Three signaling molecules [salicylic acid (SA), jasmonic acid (JA) and ethylene (E)] are involved in two major pathogen defense signaling pathways: an SA-dependent pathway and an SA-independent pathway that involves JA and E; these pathways are mostly mutually antagonistic. In general, pathogens that are controlled by SA-dependent defense responses colonize the apoplast and multiply within host tissue for several days before causing plant cell death and tissue damage (\textit{Peronospora parasitica}, \textit{Erisyphe} sp., \textit{Pseudomonas syringae}), whereas pathogens that are controlled by JA-dependent defense responses employ a virulence strategy that involves rapidly killing plant cells to obtain nutrients, and thus are often referred to as “necrotrophs” (\textit{Alternaria brassicicola}, \textit{Botrytis cinerea}, \textit{Pythium} sp., and \textit{Erwinia carotovora}) (Kunkel & Brooks, 2002). In addition to resistance mechanisms which can be induced by pathogens, plants also possess resistance tools which can be provoked upon pre-treatment with a variety of non-pathogenic organisms and which function against multiple pathogens. This general phenomenon is known as induced systemic resistance (ISR). Rhizobacteria-mediated ISR has been demonstrated against fungi, bacteria, and viruses in many plant species when the inducing non-pathogenic bacteria and challenging pathogen remained spatially separated. Some of rhizobacteria induce resistance through the SA-dependent systemic acquired resistance (SAR) pathway, whereas others do not and require JA and E perception by the plant for ISR to develop (Van Loon et al., 1998). Common in the root ecosystems, \textit{Trichoderma} spp. also can induce systemic resistance. \textit{Trichoderma} protects plants from numerous classes of plant pathogens and also frequently enhances crop productivity (Elad, 2000; Harman et al., 2004). Mutant analyses showed that rhizobacteria-mediated ISR in \textit{Arabidopsis thaliana} follows signaling pathway in which components from the JA and E response trigger a defensive state (Pieterse et al., 2001), whereas nothing is known about the role of signaling molecules in the \textit{Trichoderma}-mediated ISR. Using \textit{A. thaliana} mutants with altered hormone susceptibility or production, the purpose of this work was to study the influence of plant produced phytohormones on the basal resistance to \textit{B. cinerea} and the ability of plants to develop \textit{Trichoderma}-mediated ISR.
Material and methods

Seeds of the wild type ecotypes and mutants were provided by the Nottingham Arabidopsis Stock Center or by Arabidopsis Biological Resource Center (Columbus, OH). Seeds were sown on the autoclaved soil mix containing 70:30% volcanic gravel (tuff): peat in 7x5x5 cm pots. Following sowing, pots were held at 4°C for 2 to 5 days to enhance uniformity of germination. After stratification, pots were transferred to a growth chamber maintained at 20-22°C and 8 h photo-period/day. Short day delays the flowering and allows plants to grow larger in size. Four to 6-week-old plants were used for inoculation. Wild type and mutants were grown under the same conditions except gibberellin (GA)-deficient mutants, which were treated with GA3 solution according the instructions of collection’s holders.

A suspension (0.4%) of Trichoderma harzianum T39 (as TRICHODEX) was applied three days before inoculation as a soil drench till saturation. B. cinerea strain B4, was grown on 1/5 strength PDA in 9 cm petri dishes at 20°C for 4 days in the dark. Leaves were inoculated by placing 3 mm mycelium discs (one disc per leaf) originating from the edges of B. cinerea colonies. Inoculated plants were kept at 20°C and 100% relative humidity and 12 h photoperiod in a box covered with polyethylene. Disease severity was scored 24hr after inoculation by measuring lesion diameters and the results were expressed as lesion size in square millimeters or as lesion size in % to corresponding background line.

Table 1. Basal resistance (susceptibility) against B. cinerea in A. thaliana and Trichodex-induced ISR

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Background</th>
<th>Phenotype</th>
<th>Basal susceptibilitya</th>
<th>ISRb</th>
</tr>
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<tbody>
<tr>
<td>ABA related</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aba1-3</td>
<td>Ler0</td>
<td>ABA deficient</td>
<td>134.9*</td>
<td>-</td>
</tr>
<tr>
<td>aba2-1</td>
<td>Col0</td>
<td>ABA deficient</td>
<td>134.2*</td>
<td>-</td>
</tr>
<tr>
<td>aba3-1</td>
<td>Col0</td>
<td>ABA deficient</td>
<td>133.4*</td>
<td>-</td>
</tr>
<tr>
<td>abi1-1</td>
<td>Ler0</td>
<td>ABA insensitive</td>
<td>130.4*</td>
<td>-</td>
</tr>
<tr>
<td>abi2-1</td>
<td>Ler0</td>
<td>ABA insensitive</td>
<td>136.1*</td>
<td>-</td>
</tr>
<tr>
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<td>Ler0</td>
<td>ABA insensitive</td>
<td>143.7*</td>
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<tr>
<td>Auxin related</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>axr1-3</td>
<td>Col0</td>
<td>AUX resistant</td>
<td>182.9*</td>
<td>+</td>
</tr>
<tr>
<td>axr4-1</td>
<td>Col0</td>
<td>AUX resistant</td>
<td>117.7</td>
<td>+</td>
</tr>
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<td>Col0</td>
<td>AUX resistant</td>
<td>92.5</td>
<td>+</td>
</tr>
<tr>
<td>aux1-7</td>
<td>Col0</td>
<td>AUX, E resistant</td>
<td>134.3*</td>
<td>-</td>
</tr>
<tr>
<td>aux1-7 axr4-2</td>
<td>Col0</td>
<td>AUX, E resistant</td>
<td>111.2</td>
<td>+</td>
</tr>
<tr>
<td>ilr1-1</td>
<td>Ws4</td>
<td>IAA-leucine resistant</td>
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<td>-</td>
</tr>
<tr>
<td>rty1-1</td>
<td>Col0</td>
<td>IAA increased</td>
<td>93.9</td>
<td>NT</td>
</tr>
<tr>
<td>tir1-1</td>
<td>Col0</td>
<td>AUX transport inhibitor resistant</td>
<td>115.5</td>
<td>+</td>
</tr>
<tr>
<td>E/JA related</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ein2</td>
<td>Col0</td>
<td>E insensitive</td>
<td>132.5*</td>
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</tr>
<tr>
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<td>E insensitive</td>
<td>117.7*</td>
<td>-</td>
</tr>
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<td>Genotype</td>
<td>Background</td>
<td>Phenotype</td>
<td>Basal susceptibility$^a$</td>
<td>ISR$^b$</td>
</tr>
<tr>
<td>----------</td>
<td>------------</td>
<td>-----------</td>
<td>-------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>ein6</td>
<td>Ler0</td>
<td>E insensitive</td>
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<td>Col0</td>
<td>E over producer</td>
<td>165.4*</td>
<td>-</td>
</tr>
<tr>
<td>eto2</td>
<td>Col0</td>
<td>E over producer</td>
<td>181.1*</td>
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<td>eto3</td>
<td>Col0</td>
<td>E over producer</td>
<td>130.6*</td>
<td>-</td>
</tr>
<tr>
<td>etr1-3</td>
<td>Col0</td>
<td>E insensitive roots</td>
<td>141.2*</td>
<td>-</td>
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<tr>
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<td>Col0</td>
<td>E reduced</td>
<td>189.3*</td>
<td>-</td>
</tr>
<tr>
<td>jar1-1</td>
<td>Col0</td>
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<td>131.7*</td>
<td>-</td>
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<td>218.7*</td>
<td>NT</td>
</tr>
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<td>ga1-4</td>
<td>Ler0</td>
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<td>207.5*</td>
<td>-</td>
</tr>
<tr>
<td>ga2-1</td>
<td>Ler0</td>
<td>GA deficient</td>
<td>215.4*</td>
<td>-</td>
</tr>
<tr>
<td>ga4-1</td>
<td>Ler0</td>
<td>GA deficient</td>
<td>216.9*</td>
<td>-</td>
</tr>
<tr>
<td>spy3</td>
<td>Col0</td>
<td>GA resistant</td>
<td>154.4*</td>
<td>-</td>
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<tr>
<td>cpr5-2</td>
<td>Col0/No0</td>
<td>SA over-producer</td>
<td>99.9</td>
<td>+</td>
</tr>
<tr>
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<td>Enhanced disease susceptibility</td>
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<td>+</td>
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<td>npr1</td>
<td>Col0</td>
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<td>106.3</td>
<td>+</td>
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<tr>
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<td>Col0</td>
<td>SA insensitive</td>
<td>100.6</td>
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</tr>
<tr>
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<td>No0</td>
<td>SA insensitive</td>
<td>88.0</td>
<td>-</td>
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</table>

**GA related**

**SA related**

<table>
<thead>
<tr>
<th>Ecotypes</th>
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<tbody>
<tr>
<td>Col0</td>
</tr>
<tr>
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</tr>
<tr>
<td>No0</td>
</tr>
<tr>
<td>Ws4</td>
</tr>
</tbody>
</table>

$^a$ Values presented are lesion size in % to corresponding background line. Mutants’ lesion area designated by asterisk was significantly higher than the lesion area of corresponding wild type line, according to contrast $t$ test ($P \leq 0.05$).

$^b$ Genotypes were regarded as expressing ISR when mean lesion square in the induction treatment was significantly lower than that of control treatment (contrast $t$ test at $P \leq 0.05$).

$^c$ Lesion square of corresponding wild type line was taken as 100%. Numbers in parenthesis represent the lesion area in mm$^2$.

**Results**

The level of basal resistance to *B. cinerea* and the potential of the biocontrol agent *T. harzianum* T39 to trigger ISR responses was studied in 35 mutants originating from 4 ecotypes of *A. thaliana*. Ecotype Colombia-0 (Col0) was relatively resistant to *B. cinerea*, and *Trichoderma* application at sites spatially separated (root application) from the *B. cinerea* inoculation (leaves) resulted in 25% (up to 70%) reduction of grey mould symptoms. Ecotypes Wassilewskija-4 (Ws-4), Nossen-0 (No-0) and Landsberg-0 (Ler-0) had low level of basal resistance to *B. cinerea* and they were found to be blocked in their ability to express ISR. Plants treated with Trichodex grew faster and formed leaf rosette about 20% larger in diameter than untreated plants.
Abscisic acid (ABA)-related mutants, both deficient and insensitive, were highly susceptible to *B. cinerea*, and all mutants tested failed to express the *Trichoderma* ISR. Six out of eight auxin-related mutants were pathogen-affected similarly to their backgrounds; *axr1-3* and *aux1-7* were more susceptible. All the auxin-related mutants except *aux1-7* and *ilr1-1* expressed ISR. JA/E-related mutants included E-insensitive, E-insensitive roots, E-overproducers, E-reduced production mutant and JA-resistant one. All of them (except *ein4*) were highly susceptible to *B. cinerea* and unable to develop Trichoderma-induced ISR. Similarly, all the GA-related mutants were strongly affected by *B. cinerea* and did not express ISR. SA-related mutants were resistant to *B. cinerea* (on the level of their backgrounds) and, except *npr1-5*, expressed ISR (Table 1).

**Discussion**

Resistance of *Arabidopsis* to *B. cinerea* was reported to involve an important contribution of JA/E-mediated pathway, whereas the role of an SA-mediated pathway was only minor (Govrin & Levin, 2002; Thomma et al., 1998, 1999; Zimmerli et al., 2001). Very little is known about the role of other phytohormones (ABA, auxin, GA) in plant-pathogen interaction. Using *Arabidopsis* hormone-related mutants originating from different ecotypes, we tried to better understand the role of phytohormones in plant-pathogen interaction and in the potential of plants develop ISR.

Wild type ecotypes of *A. thaliana* differed in their susceptibility to *B. cinerea*: Col0 was rather resistant, whereas Ler0, No0 and especially Ws4 were highly susceptible, showing the possible variability in components of JA/E-response in *Arabidopsis*. Susceptible lines were unable to develop Trichoderma-mediated ISR. Earlier, 10 different ecotypes of *Arabidopsis* were screened for their potential to express rhizobacteria-mediated ISR (Ton et al., 2001): ecotype Ws was found to be blocked in its ability to express ISR and had low level of basal resistance to *P. syringae pv. tomato* (*Pst*), whereas Col and Ler were found to be ISR-inducible phenotypes resistant to *Pst*. In our experiments, Ler0 was highly susceptible to *B. cinerea* and ISR non-inducible phenotype, although data on ISR inducibility of Col and Ws corresponded with previous data (Ton et al., 2001). We suppose that ISR pathways in Arabidopsis do not depend on ISR inducing organism, and discrepancy in the data could be explained by using different lines of ecotype *Landsberg erecta*. We used line Ler0 (stock number NW20) from Nottingham collection which is the background line for Ler-derived mutants used in this work. According our data (not shown here), Ler0 and Ler1 lines differed in their susceptibility to *B. cinerea*: Ler1 was as resistant as Col0 and most likely is the ISR-inducible genotype. Maybe, this or similar genotype was used by Ton et al. (2001).

Genetic crosses between ISR inducible and ISR non-inducible *Arabidopsis* genotypes (Ton et al., 2001) revealed that ISR inducibility and basal resistance against *Pst* correlated and were inherited as monogenic dominant traits that are genetically linked in the locus *ISR1*. ISR non-inducible ecotypes exhibited reduced sensitivity to E, which suggests that *ISR1* locus encodes a component of E-response pathway that plays an important role in E-dependent resistance mechanisms (Ton et al., 2001). Based on these results, we suggest that mutants derived from ISR non-inducible ecotypes should be most probably ISR non-inducible, and the suggestion was confirmed by our data: all the Ler0-, No0- and Ws4-derived mutants were of ISR non-inducible phenotype (Table 1).

SA-impaired mutants of *A. thaliana* kept the basal resistance to *B. cinerea*, whereas most of JA/E-impaired mutants were highly susceptible, confirming that resistance of *A. thaliana* to *B. cinerea* is mediated by JA/E-pathway, while the role of an SA-mediated pathway is minor. SA-impaired mutants derived from ISR-inducible genotypes were ISR
inducible, whereas mutant npr1-5 derived from No0 was ISR non-inducible. All the JA/E-impaired mutants were ISR non-inducible. Similarly, in the experiments with rhizobacteria-mediated ISR, SA-impaired mutants had ISR inducible phenotype (except npr1-1), whereas JA/E impaired mutants were ISR non-inducible (Pieterse et al., 2001). Allele npr1-1 is not mentioned in the On-Line Catalogue of NASC, so we do not know its background and cannot discuss the possible reasons of such behavior.

At least some of ABA-, GA- and auxin-impaired Arabidopsis mutants showed changed susceptibility to B. cinerea, suggesting that components of these phytohormones’ signaling pathways play a role in resistance to B. cinerea and ISR potential. All the ABA- and GA-impaired mutants were highly susceptible to B. cinerea and showed ISR non-inducible phenotype. Although most of these mutants are derived from ISR non-inducible ecotype Ler0 and can be ISR non-inducible for this reason, some mutants are derived from ISR-inducible ecotype Col0 but still are ISR non-inducible. Recently, it was found that expression of gene tgas118, encoding a defense protein defensin, is regulated by gibberellin (Heuvel et al., 2001). Plant defensin gene is induced by pathogens via the JA/E-dependent pathway (Thomma et al., 1998), so, the high susceptibility of GA-related Arabidopsis mutants to B. cinerea suggests the link between gibberellin- and JA/E-signaling pathways. Most of auxin-related mutants of Arabidopsis were derived from Col0; they kept the basal level of resistance to B. cinerea and were ISR inducible. Auxin-resistant mutant axr1-7 was highly susceptible and ISR non-inducible, demonstrating that some components of auxin signaling pathway play a role in Arabidopsis/B. cinerea interaction and ISR potential. AXR1 locus was shown to be involved in MeJa response, providing link between JA/E- and auxin-signaling pathways (Tiryaki & Staswick, 2002). axr1-3 showing ISR-inducible phenotype in our experiments is only example of susceptible mutant being ISR-inducible. IAA-leucine-resistant mutant ilr1-1 was ISR non-inducible as parent ecotype Ws4.

Trichoderma enhanced Arabidopsis plant growth regardless of plant mutation and ISR inducibility. Enhanced plant growth and productivity was observed also on Rhizobacteria-treated hosts (Van Loon et al., 1998).

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Isolation of fungicide-resistant mutants from cold-tolerant
*Trichoderma* strains and their in vitro antagonistic properties

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Abstract: Among 128 isolates of *Trichoderma*, two cold-tolerant strains possessing excellent in vitro antagonistic properties against plant pathogenic *Microdochium nivale*, *Fusarium culmorum*, *F. oxysporum* and *Pythium debaryanum* strains were selected for this study. Seven of the 16 pesticides tested – CuSO₄, Carbendazim, Mancozeb, Tebuconazol, Imazalil, Captan and Thiram – showed significant inhibition on the *Trichoderma* strains, the minimal inhibitory concentrations were approximately 300, 0.4, 50, 100, 100, 100 and 50 µg/ml, respectively. Mutants resistant to CuSO₄, Carbendazim, Mancozeb and Tebuconazol were isolated from a *T. harzianum* and a *T. aureoviride* strain by ultraviolet light mutagenesis. The cross-resistance capabilities and in vitro antagonistic properties of the mutants were determined in the presence of sublethal concentrations of different fungicides. Carbendazim-resistant mutants showed total cross-resistance to benomyl and thiabendazole at a concentration of 20 µg/ml, while tebuconazole-resistant strains tolerated epoxiconazole at the same level as tebuconazole. A great number of fungicide-resistant strains were found to be potential candidates for application in integrated pest management.

Key words: *Trichoderma*, mutagenesis, fungicide resistance, plant pathogenic fungi, antagonism

Introduction

Plant pathogenic fungi, e.g. *Fusarium*, *Pythium* and *Microdochium* species are able to cause great losses in wheat and corn fields even below the temperature of 10°C (Nakajima & Abe, 1996). Various pesticides are used against them successfully, but these chemicals are harmful to other organisms as well.

*Trichoderma* species are imperfect filamentous fungi with telemorphs belonging to the *Hypocreales* order of the Ascomycota division. Their effective antagonistic abilities against plant pathogenic filamentous fungi are based on different mechanisms including competition, antibiosis and mycoparasitism (Manczinger et al., 2002; Papavizas 1985). Several *Trichoderma* species are effective agents to control plant pathogenic fungi, e.g. *Fusarium* (Sivan & Chet, 1986), *Pythium* (Naseby et al., 2000) and *Rhizoctonia* (Lewis & Papavizas, 1987) species, which allows for the development of biocontrol strategies.

As *Trichoderma* strains are potential biofungicides, integrated pest management strategies can be worked out based on their combined application with reduced amounts of fungicides. Furthermore, the presence of *Trichoderma* strains may have a positive effect on the germination of cereal seeds and on the growth of crops (Altomare et al., 1999). As commercial fungicides have an inhibitory effect on mycelial growth of biocontrol *Trichoderma* strains, the aim of this study was to isolate fungicide-resistant mutants for the purposes of integrated pest management.
**Materials and methods**

**Strains and culture conditions**
A number of 128 *Trichoderma* strains have been isolated from Hungarian soil samples and screened for cold-tolerance (Antal et al., 2000). Two cold tolerant isolates, *Trichoderma harzianum* T66 and *T. aureoviride* T122 were involved in this study. Plant pathogenic *Fusarium culmorum*, *F. oxysporum*, *Microdochium nivale* and *Pythium debaryanum* strains were selected from the culture collection of the Department of Microbiology, Faculty of Sciences, University of Szeged. All fungi were maintained on solid YEG medium (2 g/l yeast extract, 5 g/l glucose, 5 g/l KH₂PO₄, 20 g/l agar in distilled water). *In vitro* antagonism experiments were carried out on media containing 1 g/l yeast extract, 2 g/l glucose, 1 g/l KH₂PO₄, 1 g/l MgSO₄ and 20 g/l agar in distilled water.

**Pesticides tested and determination of their minimal inhibitory concentration values**
Minimal inhibitory concentration (MIC) values of the following 16 pesticides were determined for the two *Trichoderma* strains: MBC (carbendazim), captan, mancozeb, thiram (TMTD), tebuconazol, imazalil, fludioxonil, metalaxil, triadimefon, imidacloprid, Atrazin 500 FV, Acent A880 EC, Erunit Profi, Prometrex 50 SC, Carboxin (all dissolved in dimethyl sulphoxide, 3 mg/ml) and CuSO₄ (dissolved in distilled water, 10 mg/ml). MIC-values were determined on solid YEG medium supplemented with pesticides in descending concentrations. Presence or absence of growth was examined after 4 days of incubation. Further 10 fungicides – thiabendazole, benomyl, propiconazole mixture, epoxiconazole, penconazole, bromuconazole, diniconazole, cyproconazole, itraconazole (Orungal) and ketoconazole (Nizoral) – were involved in cross-resistance studies.

**Isolation of fungicide-resistant Trichoderma strains by UV mutagenesis**
From the conidial suspension of the wild-type strains, 10⁷ conidia were inoculated as a stripe onto solid YEG medium containing 1 µg/ml MBC, 500 µg/ml CuSO₄, 50 µg/ml mancozeb or 100 µg/ml tebuconazol and exposed to UV radiation for 30 sec.

**Cross resistance of MBC-resistant and Tebuconazol-resistant mutants**
From suspensions of MBC-resistant mutants 10⁶ conidia were put in a drop onto solid YEG media containing MBC, benomyl or thiabendazole at the concentrations of 20, 10, 5 and 2.5 µg/ml. Tebuconazol-resistant mutants were inoculated similarly onto solid YEG media containing tebuconazol, diniconazole, epoxiconazole, cyproconazole, bromuconazole, penconazole, ketoconazole, propiconazole mixture or itraconazole at the concentrations of 100, 50, 25, 12.5 and 6.25 µg/ml. Growth of colonies was examined after 5 days of incubation.

**In vitro antagonism experiments**
Mycelial disks derived from the colonies of *F. culmorum*, *F. oxysporum*, *M. nivale* and *P. debaryanum* were put onto solid YEG media containing MBC or CuSO₄ in sublethal concentrations given in Table 1. After incubation for 2-5 days (depending on the growth of the plant pathogens), mycelial disks from MBC- and CuSO₄-resistant *Trichoderma* strains were put onto the medium 30 mm apart from the plant pathogenic strains.

### Table 1. Sublethal concentrations of fungicides applied in the media

<table>
<thead>
<tr>
<th></th>
<th><em>F. culmorum</em></th>
<th><em>F. oxysporum</em></th>
<th><em>M. nivale</em></th>
<th><em>P. debaryanum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MBC (µg/ml)</td>
<td>1</td>
<td>1</td>
<td>0.8</td>
<td>0.05</td>
</tr>
<tr>
<td>CuSO₄ (µg/ml)</td>
<td>250</td>
<td>250</td>
<td>125</td>
<td>80</td>
</tr>
</tbody>
</table>
Table 2. MIC-values of fungicides on *T. harzianum* T66, *T. aureoviride* T122 and on plant pathogenic fungi (µg ml⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>MBC</th>
<th>CuSO₄</th>
<th>Mancozeb</th>
<th>Tebuconazol</th>
<th>Captan</th>
<th>Thiram</th>
<th>Imazalil</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. harzianum</em></td>
<td>0.4</td>
<td>300</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td><em>T. aureoviride</em></td>
<td>0.4</td>
<td>300</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td><em>F. culmorum</em></td>
<td>2</td>
<td>500</td>
<td>2</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>2</td>
<td>500</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. nivale</em></td>
<td>1.5</td>
<td>300</td>
<td>0.7</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. debaryanum</em></td>
<td>0.1</td>
<td>200</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Results and discussion**

**MIC-values of the distinct pesticides**

Fludioxonil, Metalaxil, Triadimefon, Imidacloprid, Atrazin 500 FV, Acenit A880 EC, Erunit Profi, Prometrex 50 SC and Carboxin showed no significant inhibition on the *Trichoderma* strains. Table 2 shows the MIC-values of seven fungicides on two *Trichoderma* strains and four fungicides on the examined plant pathogenic fungi. The MIC-values of MBC and CuSO₄ proved to be higher for three and two plant pathogenic fungi, respectively, than for the wild-type *Trichoderma* strains. Mancozeb and tebuconazole had much higher MIC-values for the *Trichoderma* strains than for the examined plant pathogenic fungi, suggesting that these compounds could be appropriate for the combination with biocontrol *Trichoderma* strains within the frames of complex integrated pest management.

The purpose of the further work was to develop *Trichoderma* strains resistant to MBC and CuSO₄, which proved to be the fungicides with the greatest inhibitory effect on the examined *Trichoderma* strains.

**Isolation of fungicide-resistant *Trichoderma* strains by UV mutagenesis**

Resistant sectors showing intensive growth were isolated and maintained on solid YEG medium. The level of resistance was checked several times and it proved to be stable in the case of the mutant strains. The numbers of stable resistant mutants isolated from *T. harzianum* T66 and *T. aureoviride* T122 are indicated in Table 3. These mutant strains seemed to be appropriate for *in vitro* antagonism experiments against plant pathogenic fungi in the presence of the corresponding fungicides.

**Cross resistance**

MBC-resistant mutants showed total cross-resistance to benomyl and thiabendazole, while tebuconazole-resistant strains tolerated epoxiconazole at the same level as tebuconazole. We suppose that similar resistance mechanisms may reveal the background of cross resistance to compounds with similar structures and modes of action.

**In vitro antagonism in the presence of MBC and CuSO₄**

Growth of the examined plant pathogenic fungi in the presence of MBC-resistant and CuSO₄-resistant *Trichoderma* strains and the corresponding fungicides in sublethal concentrations is shown in Table 4. A significant reduction of mycelial growth of the pathogen was observed in all examined mutant - plant pathogen combinations.

As the fungicide tolerance and antagonistic properties of the examined fungicide-resistant *Trichoderma* mutants are promising, they will be used in further studies aiming the development of fungicide-poliresistant strains for the purposes of integrated pest management.
Table 3. Number of stable resistant mutants isolated from *Trichoderma* strains T66 and T122

<table>
<thead>
<tr>
<th></th>
<th>MBC</th>
<th>CuSO₄</th>
<th>Mancozeb</th>
<th>Tebuconazol</th>
</tr>
</thead>
<tbody>
<tr>
<td>T66</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>T122</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 4. Growth of plant pathogenic fungi in the presence of MBC-resistant (MBC<sup>R</sup>) and CuSO₄-resistant (CuSO₄<sup>R</sup>) *Trichoderma* strains and the corresponding fungicides in sublethal concentrations

<table>
<thead>
<tr>
<th></th>
<th><em>F. culmorum</em></th>
<th><em>F. oxysporum</em></th>
<th><em>M. nivale</em></th>
<th><em>P. debaryanum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MBC + MBC&lt;sup&gt;R&lt;/sup&gt; T66</td>
<td>22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13</td>
<td>46</td>
<td>21</td>
</tr>
<tr>
<td>MBC + MBC&lt;sup&gt;R&lt;/sup&gt; T122</td>
<td>27</td>
<td>13</td>
<td>33</td>
<td>19</td>
</tr>
<tr>
<td>CuSO₄ + CuSO₄&lt;sup&gt;R&lt;/sup&gt; T66</td>
<td>33</td>
<td>29</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>CuSO₄ + CuSO₄&lt;sup&gt;R&lt;/sup&gt; T122</td>
<td>33</td>
<td>31</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are presented in percentage of growth on media without fungicides and *Trichoderma*.

Acknowledgements

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References


Protease over-production in the presence of copper by a \textit{Trichoderma harzianum} strain with biocontrol potential

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\textbf{Abstract:} The effect of copper on the production of extracellular trypsin-like proteases of \textit{Trichoderma harzianum} T66 was examined. Trypsin-like protease enzyme activities were significantly enhanced in the presence of copper ion. It was demonstrated that instead of raising the activity of previously produced enzymes, copper ions are able to induce the secretion of extracellular trypsin-like proteases. Supernatants of control and CuSO\textsubscript{4}-containing cultures with the highest enzyme activities were separated by gel filtration chromatography and enzyme activities were measured in the fractions collected. It turned out that in the presence of copper certain extracellular trypsin-like protease isoenzymes are produced in a larger degree both under inductive and non-inductive conditions. Our results suggest that the antagonistic abilities of \textit{Trichoderma} strains could be enhanced by adding certain sublethal amounts of CuSO\textsubscript{4}. Consequently, an appropriate level of crop protection could be ensured by the application of reduced amounts of copper-containing fungicides in combination with biocontrol \textit{Trichoderma} strains within the frames of integrated pest management.

\textbf{Key words:} biocontrol, copper, fungicide, plant pathogenic fungi, proteases, \textit{Trichoderma}

\textbf{Introduction}

Copper ion is a fungicide used widely in agriculture, which is due to the fact that it is a strong inhibitor of fungal growth. On the other hand, copper is necessary for normal growth in a certain small amount, as it is an important trace element.

\textit{Trichoderma} species are imperfect filamentous fungi with teleomorphs belonging to the \textit{Hypocreales} order of the Ascomycota division. Their effective antagonistic abilities against plant pathogenic filamentous fungi are based on different mechanisms including competition, antibiosis and mycoparasitism (Manczinger et al., 2002). Several \textit{Trichoderma} species are effective agents to control plant pathogenic fungi, e.g. \textit{Fusarium}, \textit{Pythium} and \textit{Rhizoctonia} species, which allows for the development of biocontrol strategies.

Integrated pest management is based on the combined application of physical, chemical and biological means of control. As copper is an effective chemical pesticide and \textit{Trichoderma} strains are potential biofungicides, integrated plant protection strategies can be worked out based on their combined application. Croatian field-trials for the control of grey mould (\textit{Botrytis cinerea}) on grapevines revealed that \textit{T. harzianum} can be mixed with pesticides based on copper sulphate and copper hydroxide (Topolovec-Pintaric et al., 1999). Mahanty et al. (2000) examined the efficacy of promising fungicides in combination with \textit{Trichoderma} for the management of foot rot of betelvine (\textit{Piper betle}) and have found that \textit{T. harzianum} along with Bordeaux mixture (1% copper sulphate) at monthly soil drenching was the best solution for controlling \textit{Phytophthora parasitica}. For the successful development of
such integrated control strategies it is very important to study the effects of copper on *Trichoderma* extracellular enzymes important for mycoparasitism. Data are available for six *Trichoderma* strains about the effects of copper-sulphate and other metal compounds on the *in vitro* activities of extracellular enzymes involved in mycoparasitism: copper slightly inhibited trypsin- and chymotrypsin-like protease and enhanced β-1,4-N-acetylglucosaminidase activities at a concentration of 1 mmol (Kredics et al., 2001a).

The aim of this study was to examine the effect of different concentrations of copper on the secretion and *in vitro* activities of trypsin-like proteases, which are among the extracellular enzymes important for mycoparasitism of *Trichoderma* strains.

**Material and methods**

**Microorganisms and culture conditions**
Strain *Trichoderma harzianum* T66 derived from the Microbiological Collection of the University of Szeged. For the induction of extracellular proteases, cultures were grown in liquid minimal medium containing skim-milk powder (1 g/l mannitol, 1 g/l NaNO₃, 1 g/l KH₂PO₄, 0.1 g/l MgSO₄ and 3 g/l skim-milk powder). Non-inductive conditions were ensured by growing cultures in liquid minimal medium (5 g/l mannitol, 1 g/l NaNO₃, 1 g/l KH₂PO₄ and 0.1 g/l MgSO₄). Media contained distinct sublethal concentrations of CuSO₄, the applied concentrations were 0, 10, 20, 30, 40, 50 and 80 µg/ml under inductive and 0, 2, 4, 6, 8, 10, 15, 20, 30 and 50 µg/ml under non-inductive conditions. Cultures were shaken on a shaker at 25°C with 100 rpm for 3 and 6 days in the case of induced and non-induced cultures, respectively. Enzyme activities were measured in the culture supernatants.

**Measurement of extracellular trypsin-like protease activities**
To provide the appropriate pH for the function of the enzymes, 50 µl phosphate buffer (pH 6.6) was added to 50 µl of the culture supernatants. After adding 50 µl of N-benzoyl-Phe-Val-Arg-p-nitroanilide (1 mg µg/ml, dissolved in dimethyl-sulphoxide) as a substrate for trypsin-like proteases, incubation followed at 25°C for 1 h and enzyme activities were measured with a Labsystems Uniskan microtiter plate spectrophotometer at a wavelength of 405 nm. Based on our experience about the susceptibility of measurements using *p*-nitroaniline substrates, the enzyme activities were considered significant, if the OD₄₀₅ values were above 0.050.

**Gel filtration chromatography**
The gel filtration chromatography was performed on a 0.9 x 60 cm K-90 column (Pharmacia) containing Sephadex G-100 gel swollen in distilled water with 1 g/l NaCl. Two ml of the supernatant was loaded to the column and 80 fractions of 0.7 ml were collected, the eluent contained 1 g/l NaCl. Activities of trypsin-like proteases were measured in the fractions after 3 h of incubation using the method described above.

**Results**

Significant trypsin-like protease activities could be measured after 3 and 6 days of culturing in the case of induced (Figure 1a) and non-induced (Figure 1b) cultures, respectively. It is apparent that the activity of extracellular trypsin-like proteases rises in the presence of certain sublethal concentrations of CuSO₄. Activity rising appears both under inductive and non-inductive conditions, but as compared with the control which contained no CuSO₄, the rate of activity rising is much higher in the case of cultures that were grown under non-inductive conditions. As copper has no significant inhibitory effect on mycelial growth at the applied concentrations, the elevated levels can not be related to differences in growth. To determine whether the presence of CuSO₄ enhances the degree of enzyme production or just raises the
activity of previously secreted enzymes, CuSO$_4$ in a concentration of 20 and 40 µg µg/ml was added to the supernatant of the control cultures grown under inductive conditions and the activities were measured. No activity rising appeared suggesting that the production and not the activity of extracellular trypsin-like proteases is influenced positively by CuSO$_4$.

![Graph](image_url)

Figure 1. Trypsin-like protease activities measured in supernatants derived from (a) induced and (b) non-induced CuSO$_4$-containing cultures of strain *T. harzianum* T66

Gel filtration chromatography profiles of trypsin-like proteases of strain *T. harzianum* T66 are shown in Figure 2. Control cultures grown under non-inductive conditions showed very low trypsin-like protease activities, which are not measurable after gel filtration. Two peaks of activity appeared in the supernatant of the induced control culture, suggesting the presence of at least two extracellular trypsin-like proteases. The production of both isoenzymes seem to rise in the presence of CuSO$_4$ both under inductive and non-inductive conditions.

**Discussion**

Copper in certain sublethal concentrations appeared to enhance the production of extracellular trypsin-like proteases both under inductive and non-inductive conditions. These results suggest that the combination of biocontrol *Trichoderma* strains with copper-containing fungicides may result in more effective integrated plant protection strategies than the application of *Trichoderma* or copper alone. Besides the addition of the inhibitory effects of the biocontrol agent and the chemical pesticide, the enhancement of *Trichoderma* protease production by copper may also contribute to the efficiency of integrated control. As copper ion has an inhibitory effect on mycelial growth with an IC$_{50}$ concentration of 0.16 mmol for strain T66 (Kredics et al., 2001a), the isolation of copper-tolerant strains seems to be important for the purposes of integration with copper-containing fungicides. Direct UV-mutagenesis with the selection at 1.6 mmol copper sulphate was ideal for the improvement of copper tolerance in *Trichoderma* strains and the resulting mutants proved to be effective *in vitro* antagonists of *Fusarium*, *Pythium* and *Rhizoctonia* strains even under copper stress.
(Kredics et al., 2001b). The results of the present study indicate that the antagonistic potential of *Trichoderma* strains can be raised by enhancing the secretion of extracellular proteases by copper, suggesting that an elevated level of crop protection could be ensured by using reduced amounts of copper-containing fungicides in combination with copper tolerant *Trichoderma* strains within the frames of complex integrated plant protection.

![Figure 2](image_url)

**Figure 2.** Gel filtration chromatography profiles of trypsin-like proteases of strain *T. harzianum* T66: induced control (●); induced+30 µg/ml CuSO₄ (■); non-induced+4 µg/ml CuSO₄ (▲)

**Acknowledgements**

This work was supported by grants F037663 of the Hungarian Scientific Research Fund and grant OMFB-00219/2002 of the Hungarian Ministry of Education.

**References**


Mycoparasitism against sclerotia of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* is widespread within the genus *Trichoderma*

Sabrina Sarrocco, Maurizio Forti, Giovanni Vannacci

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**Abstract:** One hundred twenty-six isolates of *Trichoderma* spp., belonging to the fungal collection of the Department of Tree Science, Entomology and Plant Pathology “Giovanni Scaramuzzi”, University of Pisa, were screened for their mycoparasitic ability against sclerotia of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*. Isolates belong to the *Pachybasiurn*, *Longibrachiatum*, and *Trichoderma* Sections and included *T. asperellum*, *T. atroviride*, *T. aureoviride*, *T. crassum*, *T. effusum*, *T. erina-cceum*, *T. fasciculatum*, *T. flavofuscum*, *T. hamatum*, *T. harzianum*, *T. helicum*, *T. koningii*, *T. minutissporum*, *T. oblongisporum*, *T. polysporum*, *T. rossicum*, *T. saturnisporum*, *T. sinensis*, *T. stromaticum*, *T. velutinum*, *T. virens*, *T. viride*, *Hypocrea hunua* and *Trichoderma* sp. An isolate of *Clonostachys rosea* was also tested. Some species were represented by only one strain. Mycoparasitic activity was expressed according to the percentage of decayed or infected sclerotia. Distribution of mycoparasitic ability according to the taxonomic position of *Trichoderma* spp. isolates was evaluated.

**Key words:** *Trichoderma*, mycoparasitism, sclerotia

**Introduction**

Management of the diseases caused by *Sclerotinia sclerotiorum* (Lib.) de Bary and *Sclerotium rolfsii* Sacc. with chemical measures has proved impractical, mainly because these fungi display the ability to survive in soil through the formation of dark-brown sclerotia that have strong resistance to both chemical and biological degradation. Researches have focused on developing new strategies that can provide safe and reliable means to improve crop protection. Isolates of genus *Trichoderma* have long been known for their mycoparasitic ability against sclerotia of these phytopathogenic fungi (Vannacci et al., 1987; 1989; Tsahouridou & Thanassoupolus, 2001; Sarrocco et al., 2004). The mechanisms whereby antagonists control diseases caused by sclerotial fungi may involve sclerotial degradation or interference with sclerotial germination. Inhibition of growth of the pathogen in the soil or prevention of host penetration by the pathogen, are other mechanisms that could be involved in this kind of antagonism (Benhamou & Chet, 1996). The aim of this work was to evaluate the mycoparasitic activity of more than one hundred *Trichoderma* spp. isolates against sclerotia of *S. rolfsii* and *S. sclerotiorum*. Distribution of mycoparasitic ability according to the taxonomic position of *Trichoderma* isolates was evaluated.

**Materials and methods**

**Fungal strains**

All fungal strains are deposited in the Collection of our Department. Unless otherwise stated, all fungi were maintained on PDA under oil at 4°C and grown on PDA at 24°C. *Sclerotinia sclerotiorum* (Lib.) De Bary strain 2048 was isolated in 1992 from the aerial part of tomato
plants cultivated in an experimental greenhouse in Pisa (Italy); *Sclerotium rolfsii* Sacc. strain 398 was isolated in 1987 from soil cultivated with sugar beet. Sclerotia of *S. rolfsii* and *S. sclerotiorum* were produced by inoculating 8 mm diam. disks of PDA colonized by fungal mycelium in flasks containing sterilized barley seeds soaked in water. Flasks were incubated for one month at 24°C, under 16h/8h light-darkness cycles. After incubation sclerotia were collected by sieving. *Trichoderma* spp. strains employed in mycoparasitic test belong to three Sections (*sensu* Kullnig-Gradinger et al., 2002) of the genus and included *T. asperellum* (3 strains), *T. atroviride* (9), *T. erinaceum* (1), *T. hamatum* (1), *T. koningii* (4) and *T. viride* (8) for *Trichoderma* Section; *Hypocrea hunua* (1), *T. crassum* (1), *T. fasciculatum* (1), *T. flavo-fuscum* (1), *T. harzianum* (22), *T. helicium* (1), *T. minutisporum* (1), *T. oblongisporum* (1), *T. polysporum* (1), *T. rossicum* (1), *T. stromaticum* (1), *T. velutinum* (1) and *T. virens* (3) for *Pachybasium* Section; *T. effusum* (1), *T. saturnisporum* (2) and *T. sinensis* (1) for *Longibachiatum* Section. An isolate each of *Clonostachys rosea* and *T. aureoviride* and 58 strains of *Trichoderma* spp. were also tested. “In vitro” evaluation of mycoparasitic ability of *Trichoderma* isolates

In order to evaluate the mycoparasitic ability of *Trichoderma* isolates against sclerotia of *S. rolfsii* and *S. sclerotiorum*, an “in vitro” test was performed by plating ten sclerotia of each pathogen in a Petri dish containing PDA previously colonized by each *Trichoderma* isolate. Plates were replicated twice. After 7 (*S. rolfsii*) and 14 (*S. sclerotiorum*) days of incubation at 24°C, firmness of sclerotia was evaluated. Soft sclerotia (decayed) were counted and discarded, the remaining were sterilized for 1 min in a solution of Ethanol (50%) and NaClO (1% active chlorine), washed twice in sterile distilled water, plated on PDA and incubated at 24°C. After one week plates were observed to detect the presence of *Trichoderma*. Sclerotia that gave rise to a colony of the fungus were recorded as colonized. Results are expressed as infected (the sum of decayed and colonized sclerotia) or decayed sclerotia.

**Data analysis**

Results have been analyzed pooling data at the Section level. Percentage data have been analyzed after angular transformation by the SYSTAT 10 package. Unless otherwise indicated, *P*≤0.01 was assumed as significant level.

**Results and discussion**

GLM analysis of the whole set of data (not shown) demonstrated that the effects of all sources of variability (pathogen, isolate and the interaction between these two factors) are highly significant (*P*≤0.0001) both for decayed and for infected sclerotia. In Table 1 results have been summerized at Section level. It is noteworthy the high level reached by the minimum of infected *S. sclerotiorum* sclerotia obtained by isolates from *Trichoderma* Section. GLM analysis of pooled data (excluding therefore 59 unassigned isolates and the *Clonostachys rosea* strain) (Table 2) demonstrated that there are significant differences between Pathogens both according to the percentages of decayed and of infected sclerotia. It is evident (Figure 1) the different behaviour of the two types of sclerotia according to the effect evaluated. Sclerotia of *S. rolfsii* are less prone to be infected than those of *S. sclerotiorum* but are more easily decayed than those of *S. sclerotiorum*. To better evaluate the other two sources of variation (Section and Section x Pathogen) we have to take into consideration “infection” ability.

Isolates belonging to the *Trichoderma* Section give, on the average, percentages of infected sclerotia higher than isolates belonging to the other two Sections (Figure 2). Anyway the role of Pathogen and section upon infection percentages of sclerotia will be more evident if we compare pairwise the different levels of the Pathogen x Section interaction.
Table 1. Minimum and maximum values (%) of decayed and infected sclerotia of *S. rolfsii* and *S. sclerotiorum* after facing isolates of *Trichoderma*

<table>
<thead>
<tr>
<th>Section</th>
<th>Species no.</th>
<th>Isolates</th>
<th><em>S. rolfsii</em></th>
<th></th>
<th></th>
<th><em>S. sclerotiorum</em></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Decayed Min</td>
<td>Max</td>
<td>Infected Min</td>
<td>Max</td>
<td>Decayed Min</td>
<td>Max</td>
</tr>
<tr>
<td>T</td>
<td>7</td>
<td>26</td>
<td>0</td>
<td>100</td>
<td>19</td>
<td>100</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>P</td>
<td>13</td>
<td>37</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>4</td>
<td>20</td>
<td>100</td>
<td>25</td>
<td>100</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

*T=* *Trichoderma*; *P=* *Pachybasium*; *L=* *Longibrachiatum*

Table 2. GLM analysis at Section level of data of decayed or infected *S. rolfsii* and *S. sclerotiorum* sclerotia after facing isolates of *Trichoderma*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Decayed</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogen</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td>Pathogen x Section</td>
<td>0.029</td>
<td>0.000</td>
</tr>
<tr>
<td>Section</td>
<td>0.098</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Figure 1. Decayed and infected sclerotia of *S. rolfsii* and *S. sclerotiorum* (mean values across all *Trichoderma* Sections)

Points on the same line with different letters differ for *P*<0.001. Bars = Confidential Limit for *P*<0.05.

Figure 2. Percentage of sclerotia infected by *Trichoderma* isolates at Section level (mean values for the two Pathogens)

In Table 3 significance (Tukey test) of Pathogen x Section pairwise meaningful comparisons (i.e. within Pathogens/ among Sections, lower left corner; within Sections/ between Pathogens, upper right) are reported. Pairwise comparisons give reason of significance of main sources of variation: the significant higher values of infected sclerotia caused by isolates from *Trichoderma* Section is due to the effects of these isolates on *S. rolfsii* sclerotia while differences among sclerotia types (pathogens) are due to the higher percentages of infection caused by isolates from *Pachybasium* and *Trichoderma* Sections against *S. sclerotiorum* sclerotia.
Table 3. Infected sclerotia. Significance of pairwise comparisons among Pathogen x Section interactions. Within Pathogens/among Sections, lower left corner; within Sections/between Pathogens upper right corner.

<table>
<thead>
<tr>
<th>Pathogen x Section</th>
<th>RL Mean (%)</th>
<th>RP</th>
<th>RT</th>
<th>SL</th>
<th>SP</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>RL</td>
<td>71</td>
<td>62</td>
<td>78</td>
<td>55</td>
<td>94</td>
<td>97</td>
</tr>
<tr>
<td>RP</td>
<td>62</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>78</td>
<td>ns</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL</td>
<td>55</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP</td>
<td>94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST</td>
<td>97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R = *S. rolfsii*; S = *S. sclerotiorum*; L = *Longibrachiatum*; P = *Pachybasium*; T = *Trichoderma*; ns = not significant

Mycoparasitism against *S. rolfsii* and *S. sclerotiorum* sclerotia is widespread within the genus *Trichoderma* and does not seem to be a Section character, even if significant quantitative differences among Sections could be revealed. We will move, therefore, our attention at species or isolate level. The large availability of sclerotia decaying *Trichoderma* isolates and the lack of *Trichoderma* based biopesticides targeted against sclerotia forming fungal pathogens suggest that we should pay more attention to the ecological requirements of these antagonists to be efficiently exploited in agriculture.

Acknowledgements

Authors acknowledge Prof. W. Gams (CBS, Utrecht – Netherland) for identification of some *Trichoderma* strains, Prof. C. P. Kubicek (University of Technology, Vienna – Austria) for having supplied some *Trichoderma* strains and all the students of the course of Mycology (Academic year 2003-2004) within the BS Degree in Agroindustrial Biotechnology (University of Pisa, Italy)

References


Discrimination of *Heterobasidion annosum* ISGs by evaluation of volatile organic compounds

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Abstract: *Heterobasidion annosum* represents one of the most dangerous fungi in the conifer forest of the boreal hemisphere. This fungus was differentiated into three biological species (Intersterile groups; ISGs) characterized by different host specificity, patogenicity and distribution. A fast and easy identification of the ISG has important consequences on the silvicultural decision making. However, the determination of the ISG from the morphological traits of fruit bodies or, least of all, from mycelium is usually not easy. Proton Transfer Reaction – Mass Spectrometry (PTR-MS) is a new technique proposed and realised by the University of Innsbruck, and now issued also to the market. This technique allows high performance on-line measurements for a large number of VOCs. The aim of this work was to verify the possibility to discriminate the *H. annosum* ISGs based on their volatile compound emission and to identify putative characteristic masses that may play an important role in the host specificity and in the specific antagonistic fungi among each ISGs. Twelve strains belong to the three ISGs (here indicated by F, P and S) were analysed by PTRMS fingerprinting. P ISG was definitely separated from F and S ISGs. In addition, several masses show to be significantly different among the three ISGs. ANOVA on PTR-MS peak values identified 16 significant masses out of 230. Applications and limitations of this approach are discussed.

Key words: VOCs, PTR-MS, identification, metabolic compounds, intersterile groups

Introduction

The basidiomicete *Heterobasidion* spp. have been recognised as most dangerous pathogenic fungi for the conifer forests in the Northern Hemisphere. It results in extreme destruction in pure plantations of conifers where it act like agents of root decay and white rot, but it could spread also in natural woods. Although it causes damages mainly to conifers, this polyphagus parasite has been observed on more than 200 host species, included 120 species of conifers, about 100 broadleaf species and also some non-woody plants.

In recent years numerous and important studies have been carried out on fundamental aspects of sexuality and biological cycle (Korhonen & Stenlid, 1998), as well as on biological control procedures of *Heterobasidion* (Holdenrieder & Greig, 1998). Three intersterile groups (ISGs) have been recognized in this genera. Each of them shows relatively high host specificity for Silver fir, Norway spruce and several species of pines. They are commonly identified, according with their main hosts, respectively as F, S and P ISG. These three ISGs, although morphologically very similar, result to be partially characterised from ecological, biochemical and genetical traits. The high host specificity showed by the three *Heterobasidion* ISGs is definitely the most evident ecological divergence.

Proton Transfer Reaction – Mass Spectrometry (PTR-MS) is a relatively new technique proposed and realised by the University of Innsbruck, and now issued also to the market. This technique allows high sensitivity in on-line measurements for a large number of VOCs. Recently in our laboratories we started to investigate if PTR-MS can be useful for rapid,
accurate and non-invasive characterisation of agroindustrial products as well as microorganism metabolism obtaining interesting results that confirmed the analytic/quantitative power of the technique as well as demonstrate the feasibility of an automatic PTR-MS based system for the classification of biological products (Gasperi et al., 2001; Boscaini et al., 2003; Biasioli et al., 2003a; Biasioli et al., 2003b). Interesting results have been obtained also from on-line monitoring of physiological processes in stressed plants (Fall et al., 1999). An example of application of PTR-MS fingerprint on the pathogenic ISGs of the root and butt rot fungus *Heterobasidion annosum* is presented. Moreover the preliminary data of a more systematic study on the correlation of PTR-MS static headspace fingerprinting and Quantitative Descriptive Analysis are presented.

The aim of this work was to verify the possibility to discriminate the ISGs based on their volatile compounds and to identify putative characteristic masses that may play an important role in the host specificity and in the specific antagonistic fungi among each ISGs.

**Material and methods**

The material of *Heterobasidion* spp. included in this study is composed by 12 isolates, collected mainly in Italy from 1993 to 1997. Each ISG was represented by four individual isolate. All 12 isolates were replicate three times and grown in petri dishes PDA for four weeks. The substrate was prepared in the following way: 40 g/l of Potato Destrose Agar (Difco Supplies, Detroit, Michigan).

After 4 weeks of incubation the 36 dishes plus 8 substrates white controls were opened and a circular sample of substrate with fungus 1 cm diameter and 3 mm thick for each dish was taken and transferred under sterile condition in a 22 ml amber glass vial screw cap with hole cap and septa. After 24 hours at the same room temperature of 22°C the headspace concentration of the 80 vials was analysed by PTR-MS. Measure order has been randomised to avoid memory effect. We followed the sampling procedure descibed in Biasioli et al., 2003.

**Results and discussion**

Generally single mass analysis was not able to unambiguously discriminate among the ISGs. Some exceptions, like masses 105, 89 and few others, were able to find significative differences at least between one ISG and the other two with Bonferroni multiple comparison procedure at $P \leq 0.05$. However, Multivariate Analysis combining the most informative masses, was able to clearly separate P from the other two ISGs, while S and F are still partially overlapped. This results is in agreement with previous studies with genetical and biochemical markers. Infact, it was always more difficult to separate S and F ISG, by DNA (Karjalainen & Fabritius, 1993; Kasuga et al., 1993) fatty acid and sterol profiles (Müller et al., 1995), and other metabolites.

Linear Discriminant Analysis showed allows proper identification of P ISG. The calculation was made on raw data. In this case for each pin the distance from each ISG mean is plotted. Linear Discriminant Analysis showed better performances (Figure 1) if performed on compressed data (Discriminant Partial Least Squares).

Based on literature data (Ezra et al., 2004) it is possible to tentatively associate some PTRMS peaks to some VOCs. For example M59 should be Acetone while M89 and M117 should be related to Ester fragments, e.g. 2-Methyl-Ethyl Ester (Kelsey et al., 1998; Buzzini et al., 2003). PTR-MS spectra, average are calculated for 12 samples (4 strains and 3 repetitions) of each ISG of *H. annosum*: F, S and P. ANOVA of PTR-MS peaks values calculated on the three ISGs (F, S, P) identified 16 out of 230 masses with a $P$-value $< 0.05$ (Table 1).
Table 1. Masses PTR-MS peak values sorted by decreasing significant $P$-values among the three ISGs: F, P and S. Isotopic masses and peak values under 0.5 ppb are not shown

<table>
<thead>
<tr>
<th>M/Z</th>
<th>$P$-value</th>
<th>Peak values (ppb)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>M141</td>
<td>0.0001</td>
<td>0.52</td>
<td>0.55</td>
</tr>
<tr>
<td>M105</td>
<td>0.0010</td>
<td>0.91</td>
<td>1.61</td>
</tr>
<tr>
<td>M59</td>
<td>0.0014</td>
<td>353.16</td>
<td>143.78</td>
</tr>
<tr>
<td>M137</td>
<td>0.0016</td>
<td>1.53</td>
<td>1.78</td>
</tr>
<tr>
<td>M67</td>
<td>0.0059</td>
<td>0.72</td>
<td>0.65</td>
</tr>
<tr>
<td>M87</td>
<td>0.0064</td>
<td>8.50</td>
<td>16.51</td>
</tr>
<tr>
<td>M113</td>
<td>0.0081</td>
<td>0.99</td>
<td>0.71</td>
</tr>
<tr>
<td>M117</td>
<td>0.0112</td>
<td>0.54</td>
<td>0.70</td>
</tr>
<tr>
<td>M133</td>
<td>0.0126</td>
<td>0.46</td>
<td>0.26</td>
</tr>
<tr>
<td>M89</td>
<td>0.0171</td>
<td>1.35</td>
<td>8.96</td>
</tr>
<tr>
<td>M61</td>
<td>0.0274</td>
<td>23.47</td>
<td>49.58</td>
</tr>
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<td>M107</td>
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<tr>
<td>M127</td>
<td>0.0493</td>
<td>1.43</td>
<td>3.77</td>
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</table>

These preliminary results show some evidence that PTR-MS spectral fingerprint without a priori information can provide, in relatively fast and simple way useful information to classify and describe close related taxa. Further information on the release of VOCs by *H. annosum* could be gained in order to optimize such things as inoculum concentration, medium concentration and temperature. The development of a method to accurately determine the quantity and quality of volatiles being emitted by this organism is critical for monitoring the metabolic condition of the strains in presence of an antagonistic control agent. This useful information could assist in the development of formulation bases, time and rate of application and the physical conditions most desired for the use of a biological control. Finally, how various physical and nutritional parameters influence VOC production by *H. annosum* may be determined and more easily monitored.

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References

An antifungal α-1,3-glucanase (AGN13.2) from the biocontrol fungus Trichoderma asperellum

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Abstract: Trichoderma species have been investigated as biological control agents for over 70 years due to the ability of several Trichoderma strains to antagonize other filamentous fungi, including many plant pathogenic species. Mycoparasitism is one of the main stages involved in this process. In this study, we report the antifungal activity of an exo-α-1,3-glucanase (glucan 1,3-α-glucosidase [EC 3.2.1.84], named AGN13.2, produced by the antagonistic fungus Trichoderma asperellum T32, against the strawberry pathogen Botrytis cinerea. The compatibility of AGN13.2 with other purified hydrolases and fungicides has also been tested. The antifungal activity of AGN13.2 suggests that this enzyme contributes to the antagonism of T. asperellum. In addition to its possible medical applications against dental caries, important agricultural applications can be envisaged for this enzyme.

Key words: biological control, Trichoderma, α-1,3-glucanase, antifungal

Introduction

Trichoderma strains have been widely used as biocontrol organisms for agriculture, due to their ubiquity and rapid substrate colonization, their capacity to synthesize substances inducing SAR (systemic acquired resistance) in plants, their potential for promoting plant growth, and the inhibitory effect of their antibiotics and CWDEs (cell wall degrading enzymes) against many plant pathogens. The great diversity and highly active nature of Trichoderma enzymatic systems, which includes a series of genes coding for endochitinases, N-acetyl-βD-glucosaminidases, chitin-1,4-β-chitobiosidases, proteases, endo- and exo-β-1,3-glucanases, endo-β-1,6-glucanases, lipases, xylanases, mananases, pectinases, pectin lyases, amylases, phosphohlipases, RNAses, DNAses, etc., has led to their successful use in environmental and industrial biodegradation, in composting, textiles, food and feed production, and pulp and paper treatment (Monte, 2001). The present study was undertaken to determine the antifungal activity of a new α-1,3-glucanase in T. asperellum against the strawberry plant pathogen B. cinerea.

Materials and methods

Isolates
The strain of Trichoderma spp. used in this study was T. asperellum CECT20539. Strain B98 of Botrytis cinerea isolated from strawberry was used as target fungus in the in vitro bioassay.
**Purification of AGN13.2**

The α-1,3-glucanase we present in this work (AGN13.2) was purified from supernatants of *T. asperellum* cultures in minimal medium (Penttilä et al., 1987), supplemented with 0.5% cell walls of *B. cinerea* as the only carbon source. Under this condition AGN13.2 was the only detected isozyme with α-1,3-glucanase activity. To purify AGN13.2 the filtrate of fungal cell walls-supplemented cultures was concentrated by ammonium sulfate precipitation. The concentrate was subjected to adsorption to mutan and further digestion of it. Digesting enzymes, which were released from the polymer, were subjected to chromatofocussing. Fractions within this peak were pooled, concentrated and subjected to HPLC gel filtration (Ait-Lahsen et al., 2001).

**Antifungal activity against B. cinerea**

Antifungal assays were performed in microtiter plates (Nunclon Microwell Minitray) at 25°C. Each micro-well contained 1 µL of 5x potato dextrose broth, 2 µl of a conidia suspension of *B. cinerea* (60 total spores), and 13 µl of distilled water (control) or purified enzyme in distilled water. To determine the effects of the enzyme and/or fungicide on fungal germination, the enzyme solution was added simultaneously to the conidia suspension. To determine the effects of the mixture of enzymes [protease PRA1 (Suárez et al., 2004), chitinase CHIT42 (De la Cruz et al., 1992), β-1,6-glucanase BGN16.3 (Montero et al., 2004) and α-1,3-glucanase AGN13.2] on hyphal growth, the enzyme solution was added after most spores were germinated.

**Results**

**Purification of AGN13.2**

The purification of the protein was obtained with a yield of 2%. The purified AGN13.2 was analysed by SDS/PAGE (Figure 1) with Coomasie Blue staining and a single protein band was observed, suggesting that the preparation was homogeneous. The molecular weight and the isoelectric point of AGN13.2 were 75 kDa and 6.1, respectively.

![Figure 1. Purification of AGN13.2. SDS-PAGE analysis of the different purification steps of AGN13.2. Proteins were stained with Coomassie blue. Lane 1, crude extract; lane 2, mutan digestion; lane 3, chromatofocussing eluate peak IP 6.1; lane 4, gel filtration eluate. The numbers of the right are the molecular masses of protein standards (lane M).](image)

**Antifungal activity against B. cinerea**

Regarding fungal germination of *B. cinerea*, no effects were detected after the addition of AGN13.2 by itself and combined with other hydrolytic enzymes. However the ED50 value on *B. cinerea* spore germination for the fungicide captan was reduced from 0.35 mg/mL to 0.12...
mg/mL by adding 10 µg/mL of AGN13.2 (Figure 2). Germ tube elongation of *B. cinerea* was inhibited by the mixture of an endochitinase (CHIT42), a β-1,6-glucanase (BGN16.3) and a α-1,3-glucanase (AGN13.2) (Figure 3). The antifungal activity of AGN13.2 suggests that this enzyme contributes to the antagonism of *T. asperellum*. In addition to its possible medical applications against dental caries, important agricultural applications can be envisaged for this enzyme.

Figure 2. Effects after the addition of AGN13.2 by itself and combined with fungicide captan on *B. cinerea* spore germination

Figure 3. Effects after the addition of AGN13.2 by itself and combined with other *Trichoderma* lytic isozymes on *B. cinerea* germ tube elongation
References


Montero, M., Sanz, L., Rey, M., Monte, E. & Llobell, A. 2004: BGN16.3 a novel component of the \(\beta\)-1,6-glucanase multienzymatic system in *Trichoderma harzianum*: evidences of differential evolutionary origin within the isozyme complex. – J. Biol. Chem.: in revision.


Mutual relationships between species of *Armillaria* and *Heterobasidion* on agar medium

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Abstract: Species of *Armillaria* and *Heterobasidion* are root-rot fungi that often occur in the same forests and colonize the same conifers. From the forestry point of view, *Heterobasidion* is generally more harmful whereas some common *Armillaria* species are weak pathogens only and do not attack vigorous trees. In stumps and weakened trees they are competitors of *Heterobasidion* and may thus restrict its spreading in the stand. In this preliminary work mutual relationships between different species of *Armillaria* and *Heterobasidion* were investigated on malt extract agar medium. This is the first time that *Armillaria* is used in a test as potential antagonism against *H. annosum* s.l. Four diploid isolates of *A. borealis*, *A. cepistipes*, *A. gallica*, *A. mellea* and *A. ostoyae* were paired in vitro in dual cultures with four heterokaryotic isolates of *H. annosum* s.str., *H. parviporum* and *H. abietinum*. In general, *Heterobasidion* was the stronger partner and grew slowly into the *Armillaria* colony. However, some *Armillaria* strains were antagonistic and stopped its growth. This antagonism was more a strain-specific rather than a species-specific property; however, the most strongly pathogenic *Armillaria* species, *A. ostoyae*, did not show antagonism. There were no clear differences in the behaviour of different species of *Heterobasidion*. It will be interesting to assess the relationships of *Armillaria* and *Heterobasidion* spp. in a further wood blocks experiment.

Key words: biological control, *Heterobasidion annosum*, weak pathogen, in vitro antagonism, *Armillaria* spp.

Introduction

Common occurrence of root and butt rot reduces considerably the economical value of Norway spruce in the Autonomous Province of Trento, North-Eastern Italy (La Porta, 1999). The butt rot is caused mainly by *Heterobasidion* species, but also other fungi take part in it, species of *Armillaria* in particular. However, the importance of different decay fungi in causing butt-rot in Trentino forests is not very well known (La Porta & Ambrosi, 2003).

The two species of *Heterobasidion* commonly attacking Norway spruce (*H. parviporum* and *H. annosum sensu stricto*) are strong pathogens and able to attack vigorous trees, whereas the *Armillaria* spp. are generally less aggressive pathogens. They spend mostly saprophytic way of life and attack living trees only when the trees are weakened or injured. However, the pathogenicity of different *Armillaria* species varies: some, like *A. ostoyae* and *A. mellea* are relatively strong pathogens while others, *A. cepistipes* and *A. gallica* in particular, are mainly saprotrophs. In general, the species of *Armillaria* can be regarded as being an important component of the forest ecosystem (Shaw & Kile, 1991).

Being root-rot fungi, *Armillaria* and *Heterobasidion* attack a living tree normally from the roots. Depending on the host species, they either grow up into the stem and cause butt rot (mostly in the heartwood) or they attack living tissues under the bark. In the former case the diseased tree may stay alive for decades, in the latter case it dies quickly. *Armillaria* and
Heterobasidion colonise efficiently fresh stumps of conifers (Ammillaria also of those of broadleaved trees) and use them as a nutrient base when spreading further in the stand. In fact, the man-made stumps are the main reason why these fungi are particularly common in managed forests. Mutual relationships between Heterobasidion and Armillaria have frequently puzzled forest pathologists. In particular, the possible role of the weakly pathogenic Armillaria spp. as competitors of the more harmful Heterobasidion species has aroused interest because it may have importance from the point of view of forest health.

The aim of this preliminary work was to get a better knowledge about competitive ability of five European Armillaria species against three species of Heterobasidion.

Material and methods

Four diploid isolates from each of the five European Armillaria spp: A. borealis, A. cepistipes, A. gallica, A. mellea and A. ostoyae were confronted with four heterokaryons of each of the three Heterobasidion species H. annosum s.str., H. parviporum and H. abietinum. (Table 1). The total number of confrontations was thus 240. The confrontations were carried out on malt extract agar containing 2% of Bacto Malt Extract (Difco). In later checking one supposed H. parviporum isolate (03 013) proved to be H. annosum s.str.; the number of H. parviporum and H. annosum isolates in this experiment was thus three and five, respectively.

Because Armillaria is a slow-growing fungus compared to Heterobasidion, the experiment was made as follows: a Petri dish containing malt extract agar was inoculated first with Armillaria. The inoculum was a mycelial homogenate; it was spread as a narrow line over the agar medium from one wall to the other, a little left from the diameter of the dish. A week later, when the Armillaria inoculum had grown to a solid colony (like a barrier line), the right side of the dish was inoculated with a single inoculum of Heterobasidion, ca. 2 cm from the Armillaria colony. After the mycelia met each other, the dishes were observed ca. twice a week and the position of the confrontation was marked. The incubation time was 38 days from the inoculation of Heterobasidion. At the end of the experiment the resistance of Armillaria was scored by estimating the percent area of the Armillaria colony covered by Heterobasidion. Possible antagonistic reactions were also noted.

Results and discussion

As a rule, Heterobasidion was distinctly the stronger partner in confrontations on malt extract agar medium and tended to grow over the Armillaria colony. The rate of growth of Heterobasidion over Armillaria varied from zero to total overgrowth of the Armillaria colony during the 34 days since the first confrontation of the mycelia. The ability of Armillaria to resist the overgrowth by Heterobasidion varied from strain to strain within one species, but there were differences also between the species. A. borealis, A. mellea and A. cepistipes were relatively resistant species, whereas A. gallica and A. ostoyae were distinctly weaker. A. ostoyae, in particular, was often totally or almost totally overgrown by Heterobasidion. As regards the species of Heterobasidion, H. abietinum seemed to be a little weaker species than H. annosum and H. parviporum except in the case of A. ostoyae (Figure 1). Although Heterobasidion in most cases grew over Armillaria, a number of Armillaria strains showed quite a strong antagonism against Heterobasidion and they were able to stop its advance. In such cases a narrow clear zone was formed between the confronting mycelia. Also in this respect, A. borealis, A. mellea and A. cepistipes were the strongest Armillaria species (Table 2).
Table 1. Isolates used in the interaction experiment on agar medium

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Collecting Country</th>
<th>Collecting Site</th>
<th>Host</th>
<th>Collector</th>
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<td>Ruovesi</td>
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<td>KK</td>
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<td>Salix caprea</td>
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<td>Larix decidua</td>
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<td>Trentino, Crucolo</td>
<td>Picea abies</td>
<td>NLP</td>
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<tr>
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<td>Monte Amiata, Selva</td>
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</table>

1) Collectors: CD = Claude Delatour, KK = Kari Korhonen, M F= M. Fontanari, MS = Maria Szanto, NLP = Nicola La Porta, PC = Paolo Capretti.
A. borealis  A. mellea  A. cepistipes  A. gallica  A. ostoyae

Figure 1. Resistance of five species of Armillaria against three species of Heterobasidion on malt extract agar medium. Although occasional cases of mutual antagonism occurred, the Armillaria colony was in most cases slowly overgrown by Heterobasidion. The vertical axis indicates the percentage area of Armillaria colony that was overgrown by three species of Heterobasidion at the end of the experiment. Details in text.

Table 2. The percentage of antagonistic reactions in confrontations between the species of Armillaria and Heterobasidion

<table>
<thead>
<tr>
<th></th>
<th>Heterobasidion annosum</th>
<th>H. parviporum</th>
<th>H. abietinum</th>
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<td>5</td>
<td>17</td>
<td>25</td>
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<tr>
<td>A. cepistipes</td>
<td>5</td>
<td>8</td>
<td>25</td>
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<tr>
<td>A. gallica</td>
<td>5</td>
<td>0</td>
<td>0</td>
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<tr>
<td>A. mellea</td>
<td>10</td>
<td>17</td>
<td>19</td>
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<tr>
<td>A. ostoyae</td>
<td>0</td>
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The results of this interaction experiment indicate that the Heterobasidion species are generally stronger than the Armillaria species on malt extract agar medium. Armillaria has much slower growth rate than Heterobasidion, and in most cases it is even not able to oppose the overgrowth by the latter. However, different Armillaria species vary in their ability to oppose Heterobasidion, and some Armillaria strains showed antagonism was a strain specific property. It is interesting to note that the most pathogenic Armillaria species, A. ostoyae, showed the smallest degree of antagonism. The situation may be different in the wood, where Armillaria usually protects its occupations with a strong pseudosclerotial plate against competitors. On the surface of agar medium Heterobasidion can overgrow such formations if they exist. In any case, relatively little is known about the mutual relationships of Armillaria and Heterobasidion in the wood during the decay process. The few investigations that have been made on this subject (e.g. Greig, 1962) have the drawback that the species of Armillaria
and *Heterobasidion* were not identified. Future experiments carried out in wood blocks in the laboratory or *in vivo* are needed for better understanding on the possible role of *Armillaria* in reducing the damage caused by *Heterobasidion* in coniferous forests.

**Acknowledgements**

The authors wish to thank Bresadola Mycological Group of Trento for the help in collecting and identifying *Armillaria* fruit bodies. This work was financed under the project SILE-2-Provasta by the Autonomous Province of Trento and the National Council of Researches.

**References**


Possible involvement of induced systemic resistance in sugar beet against *Cercospora beticola* by leaf treatment with *Trichoderma* sp.

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Abstract: In the attempt to consider alternative options to control of cercospora leaf spot (*Cercospora beticola*) disease we investigated a possible involvement of chitinase and peroxidase proteins in the induction of sugar beet systemic resistance by *Trichoderma* isolate BA12/86. Sugar beet cvs susceptible to *C. beticola* (Duetto and Aaron) and the partially resistant cvs (Ritmo and Faro) were treated with BA12/86 on one leaf and inoculated with the pathogen on the other leaves, 2 days after the treatment. Inoculated leaves were harvested at different times after the inoculation for protein extraction. Peroxidase and chitinase activities were determined after isoelectrofocusing (IEF) and SDS-PAGE, respectively. A peroxidase isoform (pI close to 3.6) was higher in leaves treated and inoculated (T+I); it was higher in partially resistant cvs than in the susceptible cvs, 3 days after inoculation, whereas 4-5 days after inoculation it was higher in the susceptible cvs than in the partially resistant cvs. Two chitinases (40 and 45 kDa) were present, with variable levels, in protein extracts from cv. Aaron, while a third chitinase, with higher molecular weight (50 kDa), gradually accumulated in T+I Faro plants.

Key words: *Cercospora beticola*, *Trichoderma*, PR proteins, sugar beet

Introduction

*Cercospora* leaf spot (*Cercospora beticola* Sacc.) is one of the most important and widespread fungal diseases affecting *Beta* species. It is present in most areas with warm humid climate. Control of the disease has become increasingly difficult because the fungus has developed tolerance or resistance to most fungicides. A recent study has investigated the possibility of alternative disease control of *C. beticola* with *Bacillus mycoides* (Bargabus et al., 2003). *Trichoderma* spp. are also important biological control agents, they act against pathogens trough mycoparasitism, antibiosis and competition and they induce a defence response in plants such as systemic acquired resistance (SAR).

The aim of this study has been to investigate the ability of the *Trichoderma* isolate BA12/86 (ISCI collection) applied on sugar beet leaf, to induce systemic resistance against *C. beticola*. For this purpose two families of PRs proteins, chitinases and peroxidases, accepted markers of SAR (De Meyer et al., 1998; Van Loon & Van Strien, 1999; Yedidia et al., 2000; Howell, 2003), have been monitored.

Materials and methods

Duello and Aaron sugar beet cultivars, susceptible to *C. beticola*, Ritmo and Faro, partially resistant to *C. beticola* were used. Duetto and Ritmo cvs in one experimental trial (1st
experiment), and Aaron and Faro in another one (2nd experiment) were compared. Plants were treated at 6 leaves stage with a 10-day old liquid culture in Potato Dextrose Broth (PDB), 16.6x10^6 cfu/ml of the *Trichoderma* isolate BA12/86. The treatment was applied on one leaf of each plant and *C. beticola* (1x10^5 conidia/ml) was inoculated on untreated leaves of the same plant (T+I), two days after the treatment. Non treated – non inoculated (NT-NI), non treated – inoculated (I) and treated – non inoculated (T) plants, served as controls. The experiments were carried out on 9 plants, under greenhouse conditions and repeated twice. Three, 4 and 5 days after inoculation (1st experiment) and 3, 5 and 7 days after inoculation (2nd experiment), the non treated leaves were excised and plant proteins were extracted. Peroxidase activity was determined after IEF on polyacrylamide gel within a pH range of 3-10, incubated with 0.46% guaiacol and 13 mM H₂O₂ at room temperature. Chitinase activity was evaluated after SDS-PAGE in 15% polyacrylamide gel containing 0.04% glycol chitin, treated with Calcofluor White M2R and visualized under UV (365 nm) exposure (Caruso et al., 1999). Each assay was repeated three times.

![Graphs showing peroxidase activity](image)

Figure 1. Densitometric analysis of peroxidase activity of the acidic isoform pI close to 3.6 in sugar beet leaves after leaf treatment with BA12/86 (T) and inoculation with *C. beticola* (I) at different times after the inoculation; NT-NI, non treated non inoculated plants, T+I, treated and inoculated plants. The enzymatic activity was determined by IEF on polyacrylamide gel within a pH range 3-10. On the left Duetto and Aaron cvs, susceptible to *C. beticola*, on the right Ritmo and Faro cvs., partially resistant to *C. beticola*. Duetto and Ritmo were studied in the 1st experiment, Aaron and Faro in the 2nd experiment.
Figure 2. Chitinase activity in sugar beet leaves after leaf treatment with BA12/86 (T) and inoculation with C. beticola (I) at 3, 5 and 7 days after the inoculation; NT-NI, non treated non inoculated leaves, T+I, treated and inoculated. Total proteins (35 µg per lane) from each extract were elecrophoresed on 15% polyacrylamide SDS-PAGE containing glycol chitin (0.04%). On the left Aaron cv. (susceptible to C. beticola), on the right Faro cv. (partially resistant to C. beticola). Numbers in the centre of the figure indicate the protein molecular weights.

Results and discussion

IEF assay revealed the presence of different peroxidase isoform in protein extracts from sugar beet leaves. A high acidic peroxidase isoform (pl close to 3.6) was present in T+I plants (Figure 1): its activity, 3 days after inoculation, was higher in partially resistant cvs, Ritmo and Faro, than in the susceptible ones, Duetto and Aaron, whereas, 4-5 days after inoculation an activity peak was more evident in susceptible cvs. than in the partially resistant. This isozyme activity seemed to be due to an interaction between treatment and inoculation, since it was absent or very low in NT-NI, I and T plants. Two chitinases (40 and 45 kDa) were present, with variable intensity, in protein extracts from Aaron cv., while a third chitinase, with higher molecular weight (50 kDa), mainly accumulated in T+I plants (Figure 2).

The results presented in this paper suggest a possible involvement of induced systemic resistance in sugar beet plants, being a spatial and temporal separation between BA12/86 treatment and C. beticola inoculation.

References


Going underground: nature of soil suppressiveness to *Rhizoctonia solani* in sugar beet

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**Abstract:** *Rhizoctonia solani* Anastomosis Group (AG) 2-2IIIB infects sugar beet (*Beta vulgaris* L. spp. *vulgaris*). Based on experience of growers and field experiments, soils can become suppressive to *R. solani*. The fungus may be present in the soil, but sugar beets are not infected. Knowledge of the mechanisms causing suppressiveness is essential for the development of environmental friendly control strategies to rhizoctonia root rot. A bioassay was developed to determine the level of soil suppressiveness to *R. solani*. Suppressive and conducive soil samples of commercial sugar beet fields were heated or gamma-irradiated to partly or completely eliminate the microbial flora. Two soils appeared to be suppressive to *R. solani* and the suppressiveness was reduced after irradiation or heating, indicating a biological nature of suppressiveness.

**Key words:** Thanatephorus cucumeris, conducive soils, *Beta vulgaris*

**Introduction**

*Rhizoctonia solani* Anastomosis Group (AG) 2-2IIIB infects sugar beet (*Beta vulgaris* L. spp. *vulgaris*). Decreases in sugar yield, increased tare of beets and problems with storage and processing are the result of a severe infection. Circa 15% of the total Dutch sugar beet area (100.000 ha) has severe problems with *R. solani*. Cultural practices and use of (partial) resistant varieties may reduce damage, but additional practices are still needed to control rhizoctonia disease. Based on experiences of growers and field experiments, soils can become suppressive to *R. solani*. The fungus may be present in the soil, but sugar beets are not infected. Knowledge of the mechanisms causing suppressiveness is essential for the development of environmental friendly control strategies to rhizoctonia root rot.

**Materials and methods**

A bioassay was developed to determine the level of soil suppressiveness to *R. solani*. Soils from commercial sugar beet fields that were anticipated to be suppressive or conducive were sampled at random. The soil samples were gamma-irradiated or heated (50°C and 80°C) and were infested with a sand:oat meal culture of *R. solani* (1% w/w). The bioassay was performed in a climate chamber at 23°C during the day, 15°C during the night, a photoperiod of 14 h and a relative humidity of 95%. Four weeks after sowing of sugar beet, the disease symptoms were assessed according to a disease index with 0: plant healthy to 3: plant dead.

**Results**

The disease index (DI) was significantly lower for soils 1 and 2 than for the most conducive soil when the soils were not irradiated or heated (control) (Figure 1). The DI of all soils was higher after irradiation and heating.
Figure 1. Effect of irradiation and heating on suppressive soils infested with *Rhizoctonia solani* in comparison to conducive soils. A disease index of 0 means that the soil can suppress *R. solani* completely.

**Discussion**

The suppressiveness of soils 1 and 2 was reduced after irradiation or heating, indicating a biological nature of suppressiveness. Future research will therefore focus on biotic factors. Are total microbial communities responsible for suppression or specific microorganisms? The suppression may be caused by antagonistic microorganisms (e.g. competition for nutrients and infection sites or hyperparasitism) or by toxins released by microorganisms (e.g. antibiotics, enzymes). PCR-DGGE and HPLC will be used to determine microbiological diversity and production of compounds in suppressive and conducive soils.

**Acknowledgements**

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Endophytes: A new source for multi-target biological control agents?

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Abstract: Endophytes are an interesting group of plant-associated bacteria that live inside plants and show neutral or beneficial interaction with the host plants. The structure of the bacterial community in the endosphere and endorhiza of field-grown potato was analyzed in comparison to the rhizosphere and phyllosphere by a multiphasic approach over a period of two years. Results of the cultivation-independent approach revealed that T-RFLP profiles of the two endophytic microenvironments were more closely related than those from ectophytic habitats. Using cultivation, composition, diversity and richness of bacterial antagonists confirmed specificity for each microenvironment and an outstanding and promising role of the endorhiza. In an approach to measure the biocontrol potential of isolates, a total of 2,648 bacteria were evaluated for biocontrol and plant growth promotion by a hierarchical combination of assays using the soilborne plant pathogens Verticillium dahliae and Rhizoctonia solani as target. An average of 14.4% of examined bacteria expressed antifungal properties. The strains were characterized by their antagonistic mechanisms in vitro as well as their production of the plant growth hormone indole-3-acetic acid. Complementary, the plant growth promoting effect by antagonistic bacteria was determined using a newly developed microplate assay on the basis of lettuce seedlings. Seven endophytic isolates selected according to in vitro criteria were evaluated in greenhouse and field trials regarding their efficiency to control R. solani in lettuce, sugar beet, and potatoes. In addition, they were screened for their biocontrol activity against the pathogenic nematode Meloidogyne incognita. The most promising candidates Pseudomonas fluorescens 2Re2-6 and Serratia plymuthica 3Re4-18 will be commercialized as biological control agents.

Keywords: Biocontrol, endophytes, Rhizoctonia solani

Introduction

The common soil borne pathogen Rhizoctonia solani Kühn is responsible for yield losses on an extremely wide host range of economically important crops worldwide (Anderson, 1982). Control of R. solani is difficult because of the ability to survive as sclerotia under adverse soil environmental conditions for many years, its saprophytic activity, and its extremely wide host range. Therefore, to find new strategies to control the pathogen is an important objective.

It is well-documented that an environmentally friendly alternative to protect plants against soil borne pathogens is antagonist-mediated biological control (Weller, 1988; Emmert & Handelsman, 1999). Most biological control agents (BCAs) for Rhizoctonia described previously belong to fungi, e.g. to the genus Trichoderma and only a few bacteria have been reported (Ahmed et al., 2003).
Little is known about microenvironment specificity of antagonistic plant-associated bacteria and their biocontrol potential especially for internal plant colonizers called endophytes. Endophytic bacteria have been defined by Hallmann et al. (1997) as those bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plant and, additionally, do not visibly harm the plant. It has been suggested that endophytic bacteria might interact closely with their host plants and therefore could be efficient BCAs in sustainable crop production. For example, this was demonstrated in studies which used endophytic *Bacillus* strains to control *R. solani* (Pleban et al., 1995). However, no biocontrol product on the basis of endophytes is currently on the market (Whipps, 2001).

The aim of this study was to analyze the composition of the bacterial communities, especially those from the important functional group of antagonists, and to select efficient *Rhizoctonia* antagonists. Therefore, isolates from the separate habitats defined as rhizosphere, phyllosphere, endorhiza and endosphere of field grown potatoes were investigated by a multiphasic approach over a two year period. To find out the most efficient antagonists different screening methods were used or developed, and hierarchically combined. Selected antagonists were evaluated under greenhouse and field conditions to control *Rhizoctonia solani*.

**Materials and methods**

**Isolation of bacteria**

Potato plants cv. Cilena were sampled at the experimental station of the Institute for Plant Diseases, Bonn University, in Bonn-Poppelsdorf (Germany). Roots with adhering soil and leaves were collected and then transported to the laboratory. For the isolation of endophytic bacteria, roots and leaves were surface sterilized in 1.5% sodium hypochlorid solution for 3 min followed by three washes in sterile tap water. The plant material was imprinted on nutrient agar as sterility check. Only samples were no bacterial growth occurred in the sterility check were included in further analysis. All samples were homogenized with mortar and pestle, serially diluted with sterile 0.85% NaCl solution and plated onto R2A medium (Difco).

**DNA extraction and T-RFLP analysis of bacterial communities**

Bacterial pellets recovered from different microenvironments were obtained by differential centrifugation and stored at -70°C. From these pellets total DNA was extracted using FastDNA Spin Kit for Soil (rhizosphere, endorhiza and endosphere) and FastDNA Kit (phyllosphere) (Bio101). T-RFLP analysis based on 16S rDNA fragments from community DNA amplified with primer 799f fluorescently labeled with 6-FAM and with primer 1525r were carried out as described by Berg et al. (2004).

**Screening of bacteria for antagonism towards *Rhizoctonia solani***

All bacterial isolates were screened for their activity by a dual culture in vitro assay on Waksman agar (Krechel et al., 2002).

**Screening for plant growth promotion on lettuce seedlings**

Surface sterilized (1% NaOCl, 5 min) lettuce seeds 'Daguan' (S 5601, Syngenta) were treated with different bacterial strains at 10^5 cfu/ml and compared with a control of 10 µl of distilled water. The plants were incubated for two weeks (22/16°C, 16/8 h day/night, and artificial lights) in a chamber (Percival Scientific) and analyzed according to Faltin et al. (2004).

**Screening for biocontrol activity**

The effect of the bacteria to inhibit symptom expression by *R. solani* on lettuce leaf discs cv. Daguan was tested according to Fiddaman et al. (2000). Additionally, the ability of bacterial
isolates to inhibit damping-off disease caused by R. solani (RHI Ben 4, AG 4) on sugar beet seedlings cv. Dorena was tested after seed bacterization according to Faltin et al. (2004).

Field experiments
The bacterial BCAs were tested against black scurf diseases on potato ‘Exquisa’ under field condition in Großbeeren (lat. 52° N) without and after an additional artificial infestation with R. solani (isolate Ben 3). Additionally, the effect of the BCAs on bottom rot on lettuce was tested on two experimental fields (100 x 24 m) of the Institute of Vegetable and Ornamental Crops located in Golzow and Großbeeren (Germany), both naturally infested with R. solani. Details of the experiments are published by Grosch et al. (2004).

Results and discussion

Microenvironment specific composition of bacterial communities in potato
For cultivation-independent analysis, total community DNA was extracted from the microbial pellet recovered from the four microenvironments. 16S rDNA fragments amplified by PCR were analyzed by T-RFLP. All T-RFLP profiles displayed high community diversity. A cluster analysis based on all T-RFLP profiles clearly demonstrated the high specificity in different microenvironments as shown in detail by Berg et al. (2004). In most cases profiles of the same microenvironment clustered together. However, the branches of the microenvironments showed a different relationship to each other depending on the sampling times studied. In young plants endosphere and phyllosphere were grouped together, whereas the endosphere in flowering plants were clearly separated from the other microenvironments. In senescent plants both endophytic communities displayed similarities and could not be clearly distinguished. At this sampling point both the endosphere and the endorhiza were separated from the two other microenvironments.

Microenvironment specific occurrence of Rhizoctonia antagonists in potato
A total of 2,648 bacterial isolates were screened for their antagonistic ability to suppress growth R. solani in an in vitro dual culture assay. A total of 301 isolates were found antagonistic against the pathogen. Although similar numbers of isolates were tested from each microenvironment the proportion antagonists varied depending on microenvironment and sampling time. The lowest variability was found in the rhizospheres where the proportion of antagonists varied between 3 and 13%. In contrast, the highest variability was observed in the phyllosphere. While in the rhizosphere and endosphere antagonistic bacteria occurred in all samples, samples of the phyllosphere and endosphere sometimes yielded no antagonists at all. The number of isolates with antifungal activity against R. solani was highest for the phyllosphere (137), followed by the endorhiza (80), rhizosphere (66), and endosphere (18). During the vegetation period, the proportion of antagonists towards R. solani decreased.

Microenvironment specific diversity of Rhizoctonia antagonists in potato
The majority of Rhizoctonia antagonists were identified at the species or genus level by FAME-GC and/or sequencing of the 16S rRNA (Berg et al. 2004). Altogether, 38 bacterial species could be identified. The highest number of species with antagonistic properties was isolated from the rhizosphere (24) and endorhiza (13) while 14 and 10 species were found in the phyllosphere and endosphere, respectively. Richness and diversity indices confirmed the specificity of microenvironments and showed a high diversity of bacterial antagonists in the rhizosphere. Two species were obtained from all microenvironments, i.e. Bacillus pumilus and B. subtilis. Microenvironments which shared the most species were the endorhiza and rhizosphere with ten species A low similarity regarding antagonistic species was found between the endorhiza and the endosphere. Only two common bacterial species were detected...
there. In addition to the two bacterial species which occurred in all microenvironments, *Agrobacterium tumefaciens*, and *Pseudomonas fluorescens* were detected. In the ectophytic microenvironments, five additional species occurred in common: *Bacillus amyloliquefaciens*, *Pseudomonas chlororaphis*, *P. putida*, *Staphylococcus epidermidis*, and *S. pasteuri*. The most frequently isolated bacteria were characterized as *Bacillus pumilus* (63 isolates), *Pseudomonas putida* (53 isolates), and *B. subtilis* (44 isolates).

**Screening for the most efficient Rhizoctonia antagonists**
A hierarchical strategy and assessment was used to characterize and assess potato-associated bacteria for biocontrol against *R. solani* as described by Faltin et al. (2004). Altogether, the strategy consisted of six different steps. Firstly, the antagonistic activity against two *Rhizoctonia* strains was evaluated. In a second step, antagonistic mechanisms as well as mechanisms of plant growth promotion were analyzed in vitro followed by the identification of isolates. The biocontrol activity ad planta was investigated using lettuce and sugar beet as host plants. All selected isolates were genotypically characterized by a molecular fingerprint. Finally, the best candidates were selected according to their efficiency in all assays (Table 1). With exception of *B. subtilis* 1Pe4-14, which was isolated from the phyllosphere, all effective isolates originated from the endorhiza. The highest score of 23 was achieved by *P. trivialis* 3Re2-7 which is also active against *Meloidogyne incognita* (Faupel 2003).

Table 1. Effect and assessment of endophytes in biocontrol and plant growth promotion

<table>
<thead>
<tr>
<th>Strain</th>
<th>Effect on Fungus</th>
<th>Effect on Seedling</th>
<th>Assessment</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>DS³</td>
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<tr>
<td><em>Pseudomonas trivialis</em> 3Re2-7</td>
<td>6</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td><em>Serratia plymuthica</em> 3Re4-18</td>
<td>6</td>
<td>6</td>
<td>7</td>
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<tr>
<td><em>P. fluorescens</em> 2Re2-6</td>
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<td>0</td>
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<tr>
<td><em>Bacillus subtilis</em> 1Pe4-13</td>
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<td>3</td>
<td>7</td>
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<tr>
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<tr>
<td><em>P. corrugata</em> 5Re4-12</td>
<td>6</td>
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</tbody>
</table>

¹ Antagonistic activity in dual culture, scale 0-6; ² plant growth promotion lettuce seedlings, scale 0-6; ³ disease severity lettuce, scale 0-7; ⁴ disease severity sugar beet, scale 0-7.

**Evaluation of Rhizoctonia antagonists**
Altogether, two of the endophytically living bacterial strains, *Serratia plymuthica* 3Re4-18 and *Pseudomonas fluorescens* 2Re2-6, were able to reduce symptoms caused by *Rhizoctonia* on potato and lettuce under greenhouse and field conditions. While *Pseudomonas fluorescens* was the most efficient one in the pathosystem *R. solani* – potato, the endophyt *Serratia plymuthica* 3Re4-18 showed the best results in controlling the pathogen in lettuce. In climate chamber experiments, disease severity (DS) by *R. solani* could be reduced by B1 52% on average on potato. Also under field conditions, the *Pseudomonas* reduced significantly the DS as well as under low and high disease pressure. The proportion of tubers highly infested with *Rhizoctonia sclerotia* were reduced in the bacterial treatments. Treatment with *Serratia plymuthica* resulted in the most efficient inhibition of *R. solani* on lettuce. The reduction of DS was correlated with enhancement of plant growth of lettuce. Although a variability of the biocontrol effect in the experiments repeated independently was observed, the effect was to be seen in the majority of trials (Grosch et al., 2004). Therefore, both endophytic candidates are
promising biological control agents.

References


Novel understanding of the biocontrol mechanisms of *Trichoderma*, a mycoparasite and an opportunistic avirulent plant symbiont

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*Trichoderma*-based biofungicide are a reality in commercial agriculture, with more than 50 formulations registered worldwide as biopesticides or biofertilizers. Several research strategies have been applied to identify the main genes and compounds involved in the complex, three-way interactions between fungal antagonists, plants and microbial pathogens. Proteome and genome analyses have greatly enhanced our ability to conduct targeted and genome-based functional studies. We have obtained reproducible 2-D maps of the entire fungal proteome in various conditions of interaction, which permitted the isolation of many proteins related to specific functions. Many differential proteins from several biocontrol strains of *Trichoderma* spp. during the *in vivo* interaction with different plants and/or several phytopathogenic fungi have been isolated and analysed by MALDI-TOF. Relevant genes have been cloned and specifically inactivated, to demonstrate their function in biocontrol and induction of disease resistance. GFP-based reporter systems with interaction-inducible promoters allowed the characterization of regulatory sequences activated by the presence of the pathogen or the plant. From extensive cDNA and EST libraries of genes expressed during *Trichoderma*-pathogen-plant interactions, we are identified and determined the role of a variety of novel genes and gene-products, including ABC transporters specifically induced during antagonism with other microbes; enzymes and other proteins that produce or act as novel elicitors of Induced Resistance in plant and promote root growth and crop yield; proteins possibly responsible of a gene-for-gene avirulent interaction between *Trichoderma* and plants; mycoparasitism-related inducers released from fungal pathogens and that activate biocontrol in *Trichoderma*; fungal promoters specifically induced during mycoparasitism and plant colonization; plant proteins and a novel phytoalexin induced by the presence of the fungal antagonist; etc. We have also transgenically demonstrated the ability of *Trichoderma* to transfer heterologous proteins into plants during root colonization. Finally we have used GFP and other markers to monitor the interaction *in vivo* and *in situ* between *Trichoderma* and its host(s) (the fungal pathogen and the plant).
Studies on induced resistance against fire blight (*Erwinia amylovora*) with different bioagents

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Abstract: Three different bioagents (BION, BioZell-2000B, an etheric oil from *Thymbra spicata*, and the antagonistic bacterium *Rahnella aquatilis* Ra39) were tested for their biocontrol effect against fire blight (*Erwinia amylovora*) and for resistance induction activity. The experiments were carried out under controlled climatic conditions in a greenhouse. M26 apple rootstock was used as host plant. Furthermore, enzyme activities of β-glucosidase and PR-protein (chitinase) were estimated as markers of resistance in physiological studies. The treatment with BION, BioZell-2000B and Ra39, resulted in a marked reduction of the disease index, up to 64%. This was correlated with a decreasing effect on the growth of bacteria, up to 64% during the course of infection. In the physiological studies significant changes in the activities of β-glucosidase and chitinase were found after treatment of all three bioagents. Thus the biocontrol effect was correlated with a resistance induction in the host plant of apple rootstock.

Key words: bioagents, induced resistance, fire blight

Introduction

Induced systemic resistance as a general phenomenon in plants were already described with plants pathogens. In compatible host parasite interaction bacterial pathogens, a-biotic agents and stress have been largely used to induce systemic resistance against bacterial diseases (Goodman et al., 1986). In the following study the three bioagents Bion, BioZell-2000B and the antagonistic strain Ra39 of *Rahnella aquatilis* were assayed on their potential effect against fire blight on the highly susceptible apple rootstock M26.

Material and methods

Plant material and application of treatments

As host plants, M26 rootstock plants were grown in pots under greenhouse conditions (25°C±5 and 5000-14000 lux) until shoot length of 20-30 cm. BION®, Bio Zell 2000-B were used at a concentration of 0.05% (diluted with tap water) as inducing agent by spraying on leaves at 48 h before inoculation. Spraying solution of the strain Ra39 was prepared from the pellet of liquid cultures in saline to an optical density of 0.2 at 660 nm. Control plants were treated similarly with tap water.

Inoculation

The youngest two leaves of the shoots were cut on the tip and inoculated by dipping into a suspension of 1x10⁸ CFU/ml of a virulent strain of *Erwinia amylovora* Ea (7/74) (Zeller & Meyer, 1975).
**Determination of bacterial development**

For the determination of bacterial multiplication, samples of shoot tips (ca. 1 g) were homogenised with a 0.06% NaCl solution. From each homogenate a dilution plating (1:10 to 1:1000) was made on modified Miller-Schroth medium Zeller and Brulez (1987) and incubated for 2 days at 27°C.

**Determination of symptom development**

This was evaluated according to a rating system from 0 (no symptom) to 10 (whole shoots infection). From the data disease index (%) was calculated. Baysal and Zeller (2000).

**β-Glucosidase (β-GL) activity**

Half ml supernatant was incubated for 5 min with 1.5 ml Sörensen phosphate buffer pH 6.5 (6.8 g KH₂PO₄ and 8.99 g Na₂HPO₄ x 2H₂O are dissolved in 1000 ml water, after addition of 0.372 g/l EDTA the pH is adjusted to 6.5) and 0.5 ml 5 mM p-nitrophenylglucopyranosid at 30°C and measured at 400 nm (Zeller, 1985). β-Glucosidase activity = mM p-Nitrophenol / mg protein

**Chitinase activity**

Chitinase activity was determined using the method described by Wirth & Wolf (1992). High polymeric carbomethyl-substituted chitin labeled covalently Remazol Brillant violet 5R (CM-chitin-RBV- F. Loewe Biochemica) was used as substrate for chitinase.

**Results and discussion**

Disease index of fire blight in shoots of M26 rootstock plants was suppressed by BION®, BioZell-2000B and Ra39 up to 64, 52 and 59%, respectively (Figure 1). This was correlated with a decreasing effect on the growth of bacteria up to 64, 50 and 64% during the course of infection (Figure 2), which could be detected also in studies of other authors (Brisset et al., 2000; Baysal et al., 2002).

![Figure 1. Disease index of fire Blight in shoots of M 26 rootstock after treatment with BION, BioZell-2000B and Ra39 (n= 8)]
Increase in β-glucosidase and chitinase activity in several studies mentioned as PR-protein and associated with SAR(Systemic Acquired Resistance) (Kessmam et al., 1994), were found after treatment with BION®, BioZell-2000B® and Ra39 in both non inoculated and inoculated shoots after 6 and 4 days respectively. In non inoculated shoots, β-glucosidase activity increased by 133, 33 and 100% respectively after 6 days application (Figure 3). Also chitinase activity enhanced after the same period by 156, 108 and 174% (Figure 4). Moreover in inoculated shoots β-glucosidase activity increased by all treatments by 100, 67 and 83% after 4 days application respectively (Figure 5). Also chitinase activity was enhanced after the same period by 98, 32 and 63% 6 dpi (Figure 6). This physiological changes can be considered as a resistance inducing reaction, which is generally appearing after application of resistance inducers (Schönbeck et al., 1980).

Figure 2. Growth of Erwinia amylovora in shoots of M26 rootstock after treatment with BION®, BioZell-2000B and Ra39 (n = 2)

Figure 3. Effect of treatment with BION®, BioZell-2000B and Ra39 on β-Glucosidase activity in inoculated shoots of M 26 rootstock (n = 3)
Figure 4. Effect of treatment with BION®, BioZell-2000B and Ra39 on activity of chitinase in non-inoculated shoots of M 26 rootstock (n = 3)

Figure 5. Effect of treatment with BION®, Bio Zell-2000-B and Ra39 on ß-Glucosidase activity in non-inoculated shootsof M26 rootstock (n = 3)

Summarizing the results of this study, it can be demonstrated that all three bioagents were able to induce a defence reaction against fire blight in the tested host plant, a reduction in symptom development was correlated with a decrease in the bacterial multiplication and enzymatic changes indicated an induced resistance reaction through all bioagents.
Figure 6. Effect of treatment with BION®, BioZell-2000B and Ra39 on activity of chitinase in inoculated shoots of M 26 rootstock (n = 3)

References


Round Table:
What will be the future for BCAs?
Risk related to BCAs: reality or phantom risk?

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Abstract: This paper originates from a round table discussion on the future of BCAs. It analyzes the constraint to research, development, registration and finally commercialization of microorganisms to be used for biological control of pest and diseases of crops and weeds through phantom risks. Some possible action to avoid the constraint are suggested.

Keywords: crop protection, constraints

Introduction

Microorganisms, filamentous fungi, yeast and bacteria, have a potential to be used as antagonists, and/or parasites to control pests and diseases of crops or as mycoherbicides to control particular weeds. Several are reported to be active and some are already commercialised. They are living organisms, which have the capacity to multiply, spread and colonize new non-target sites, they can evolve and therefore adapt to new environmental conditions. These intrinsic capacities, which differentiate them from control measures of pests and diseases, based on natural and synthetic chemicals raise also the question if there may not be unwanted and permanent side effects. During research and development of Biocontrol agents (BCAs) some potential risks can be analysed and clearly excluded or proven and appropriate action can be taken. However, often R&D is confronted with opinions on risks posed by BCAs which can not clearly be stated, such risk is defined as phantom risks. In this contribution, some cases will be exemplified and discussion will be opened, so to make aware the R&D which problems they may encounter during commercialisation of a BCA.

What is a phantom risk?

Phantom risks are risks which are discussed in the media and and by the general public without that the scientific research delivers clear results and conclusions (studies pro and contra) or the results are not convincing.

Phantom risk is the expression of insecurity of the society and is related to the lack of trust in the scientists. It can have a relevant percussion on the acceptance of a technology by the general public. Clear examples are the “electrosmog”, the emission of radio waves by the cellular phones and creation of new “supervirus” in transgenic plants. Some phantom risks were often negated by scientists and experts, which later proved to be real as “Feedstuff of animal origin can not be the cause of any animal disease” (expert’s opinion), it transmitted the “mad cow” disease”. Examples are numerous and each novelty is always confronted with phantom risks. Often a phantom risk for a novelty lays in the abandoning of a use, a strategy or a tool which the novelty substitutes. Some appear today as ridiculous and may have appear so to an expert public in the past as the warning end of the 19 century that the speed of the trains may render the passenger insane because our brain can not deal with such a rapid change of images.
Potential BCA related phantom risks

A few examples can foster our point: Tolerance of the presence of a pathogenic fungus on food carries the risk of mycotoxin contamination; therefore we should not abandon chemical control for a control strategy which does not completely eliminates a pathogenic fungus. A statement made in relation to apple scab.

BCAs may produce toxic substances on particular food and/or under particular conditions, as it is well known that the production of metabolites (quantitatively and qualitatively) depends on the substrate and environmental conditions. BCAs may alter product e.g. shorter shelf life, consistency or taste. It is a particularity of phantom risks that they are brought into discussion at surprising moments and it is impossible to preview them.

Phantom risk and experts

Scientists often negate the existence of phantom risk for several reasons: “Experts” on the subject deny the relevance of studies contrary to their opinion; “Experts” often associate phantom risk to lack of knowledge and understanding. As phantom risk shed doubts on the expert’s opinion, the expert will often react emotionally and can and will not enter a discussion but will try to solve phantom risk by preaching his/her doctrine.

How to solve risks?

Real risk can be described and therefore solutions can be found, such as: abandoning R&D of the potential BCA-organisms (most frequent solution); search for similar organisms without the unwanted property; eliminate genes coding for unwanted property; change application timing, method. The possible action are logic and economical based and can be scientifically underpinned. For Phantom risks no scientific solution can be found nor can they lead to logical solutions.

Phantom risk and policy makers

Policy makers have to preview and avoid potential public health hazard and environmental negative impacts. Policy makers are “non-experts”. They are “safe-policy” oriented. Therefore, for policy makers phantom risk are real except their trust in the expert lead them to negate the presence of a particular phantom risk. However, the past experience of a few highly publicist mistakes of scientists induces policy makers to a very cautious approach.

Phantom risks and R&D of BCAs

Presence of phantom risk will hamper BCA R&D. They may lead to a basic negative feeling in research financing agencies toward BCA R&D project. Fear of critic to waist money. Phantom risk will also increase “burden of registration” as data may have to be delivered which the scientist thinks that is evident or irrelevant. Still these obstacles can be overcome with clear and open information. More worrying is that phantom risk can seriously hamper the application of BCA in practice. Rumours, uncontrolled voice information can spread and insinuate doubts. Non-scientific information material, journals, web pages are full of examples suggesting risks which we define phantom risks. They can be placed with a clear often economical scope, out of a general phobia of novelties or misinterpretation.
How to avoid phantom risks

First step is to check early for potential phantom risks. Do not suggest them to “non experts”. Do not negate that contrasting scientific opinion may exist. Present scientific evidence and not dogmas. Build confidence. Phantom risk can surge to a problem but can also deflate, mostly these processes are parallel to the real or perceived advantage the consumer has or has not. A consumers will disregard any phantom risk (even a real risk) if the novelty brings advantages, contrarily he will perceive a phantom risk as real if he has no advantage or worse if a unlinked party may draw an advantage. This leads to the suggestion that R&D of BCAs has to make clear what are the advantages for the consumer and environment of the use of BCAs. Communicating the advantages is therefore an essential task.
Transferring scientific results into practice – experience and problems

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Introduction

Over the past years, farmers in The Netherlands have tested and implemented new practices in arable crops and flower bulbs in a project funded by the Dutch Ministry of Agriculture. In 2003, a similar, new project was funded, aimed at all major crops including glasshouse vegetables and ornamentals. It is basically an implementation project in which all state-of-the-art knowledge of growers, researchers and consultants is combined. This paper will describe the set-up of the work and the first experiences in glasshouse crops.

The project is run in collaboration with agricultural organizations and consultants from private companies. It aims at implementation of growers’ knowledge combined with scientific results and dissemination of tested knowledge and experiences through the growers’ network. Active communication focuses on distribution of technical information as well as on increasing acceptance. Milestones that should be achieved at the end of the project in 2006:

– most recent knowledge is widely tested by growers
– it is clear which measures are effective and feasible
– questions and problems have been discussed with policy makers
– agricultural organizations and consultants actively spread knowledge on and experience with effective and feasible measures
– effective and feasible measures are broadly used by growers in the groups and others.

Groups of growers

In 2003, four groups of 5-10 “learning” growers were formed, one for cucumber, one for tomato, one for rose and one for chrysanthemum. These crops were chosen for several reasons. In cucumber, severe problems remain with powdery mildew control and *Mycosphaerella*, and because of the relatively short cropping period it is hard to establish effective populations of natural enemies of pests. In tomato, recent research has shown interaction between diseases and their effect on yield, and the energy-saving climate regimes may promote diseases like *Botrytis*. In rose and chrysanthemum, only part of the growers uses natural enemies for pest control. In 2004, a group of potted plant growers will be formed; in 2005 one for mushroom growers will follow. The groups meet several times per year and in between they are visited by the researchers from Applied Plant Research (PPO).

Work in the groups of growers

For each grower in each group, a crop protection plan is designed before the start of the crop. The plan is the result of discussions between the grower, his regular consultant on crop protection (usually from a crop protection company) and the researchers from PPO. The researcher(s) introduce the most recent developments in integrated control strategies as described elsewhere in these proceedings (Dik *et al.*). During the cropping period, the growers...
register the input of chemical and natural pesticides, natural enemies and time spent on cropping measures aimed at controlling pests and diseases, for example removal of infected plant parts etc. Yields are recorded as well, and at the end of the cropping period, the economic result as well as environmental impact is calculated. With these results, a new crop protection plan is drawn up for the next crop. The idea is that a stepwise implementation of best practices will be achieved (Figure 1).

**Figure 1. Schedule of step-wise implementation of best practices. The cycle is run once per crop.**

**Communication outside the groups**

Simultaneously with the work in the groups, a lot of effort is aimed at dissemination of results to other growers and convincing them to also implement the strategies that prove to be feasible. Also, we try to involve other parties in glasshouse horticulture in the project so that they will support the new strategies as well. First of all, we look for participation of crop protection companies by a) including them in designing the crop protection plan of the growers in the groups and by b) meeting with groups of consultants to discuss the strategies they advise to growers and compare them to our results. Grower’s associations are involved by reporting on the project on a regular basis.

The main goal of the communication is creating a positive attitude towards change. For a large part, this is achieved by showing results and by dissemination of technical information.

We develop leaflets with information for the major pests and diseases for each crop and this information is also put on the (Dutch) website www.telenmettoekomst.nl. Interviews with growers are published describing their experiences. Furthermore, we organize excursions to glasshouses of participating growers.

**Experiences 2003 – 2004**

A researcher from PPO is heading each group. These researchers were chosen for leadership qualities and were trained in managing processes of change. The researcher has to be flexible in order to follow the developments in the group. For example, the groups decide on the frequency of the meetings and the subjects to be discussed. Each group has growers with different attitudes towards integrated control. It is important to stimulate discussions between growers to promote acceptance of the strategies. The process needs a careful balance between being too ambitious and being too cautious. It is important to create a bond between the members of the group so that they feel free to share their successes as well as their failures and also feel free to mention the use of non-registered products.
So far (June 2004) the project is going according to plan. No major obstacles have been encountered. It was fairly easy to find growers and the atmosphere in the groups is good. The next challenge will be the interaction with the consultants from private companies who have their own strategies. There is a lot of competition between these companies and some are afraid their competitive strength will be diminished. This can be addressed by the fact that not one single strategy will be developed for each crop and the extra value of the consultant in the long run will be to help the growers to combine strategies into the optimal one for that specific situation. Grower’s organizations have been involved in every step in the project and are supporting the overall goal. However, some fear exists that strategies that have been shown to be feasible will somehow become new laws. This doesn’t have to be a real problem if the strategies can easily be implemented by all growers.

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EU registration problems and possible solutions

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The Council Directive 91/414/EEC of July 1991 concerning the placing of plant protection products on the market sets out a Community harmonised framework for authorization, use and control of plant protection products. It is a dual system whereby the Community evaluates active substances and the Member states evaluate and authorise products containing them. A basic principle of the directive is the development of a positive list (Annex I) of active substances that are acceptable for the environment, and for human and animal health. The Directive provided for a twelve-year programme of evaluation of the active substances already on the market at the time of its entry into force in July 1993 (the “existing” active substance).

The Commission divided the review programme for these substances into four groups. Any other substance presented after July 1993 is considered as a “new” substances. Both new and existing substances are evaluated in parallel. The fourth list comprises the remaining substances identified as being of lower concern and to which other data requirements than those for the others lists, might apply. The list includes microbial pesticides, substances already authorised in foodstuffs, plant extracts, animal products, substances used in organic farming, rodenticides, storage product and commodity chemicals. The Regulatory framework in which micro-organisms including bacteria, fungi and viruses are evaluated is the Commission Directive descending from the Council Directive 91/414/EEC and relative amendments. Annexes IIB and IIIB to Directive 91/414/EEC set out the requirements for the dossier to be submitted by an applicant respectively for the inclusion of an active substance consisting of micro-organism or viruses in Annex I to that Directive and for the authorisation of a plant protection product based on preparations of micro-organism or viruses.

It is appropriate to differentiate data requirements for chemical substances and micro-organisms in some respects, since several requirements are specific for micro-organism, such as the infectivity and pathogenicity. Since experience was gained during the evaluation of a number of new active substances consisting of micro-organism, the Commission Directive 2001/36/EC has amended Annexes IIB and IIIB to the Directive 91/414/EEC regarding the data requirements for microbial active ingredients and formulated plant protection products.

A fourth stage of work was provided for in Regulation (EC) N°. 1112/2002 of June 2002. Producers wishing to secure the inclusion of the active substances covered by this stage in Annex I to Directive 91/414/EEC have undertaken to provide the necessary information. Many of the active substances covered by the fourth stage of the review are produced in small volume for specialist purposes. Some are important in organic or other low-input farming systems and may be expected to present a low risk in terms of human and environmental protection. A modified approach is required for the fourth stage of the working programme of work to reduce the risk that large numbers of active substances will be withdrawn only for economic reasons.

Assometab is an Italian association for the production and commercialisation of agricultural product mainly used in the integrated and organic farming. The Companies
associated to Assometab see several problems on the process of evaluation of the plant protection products based on micro-organism. These are mainly related to the preparation of the technical dossier, definition of the task force agreement and the deadline for the submission of the dossiers.

Specific issues in the registration of bio-pesticides are related to the process of the evaluation of the active substance. So far only three active substances have been included in Annex I and the average time needed was close to six years. The reasons may be related to lack of a specific regulation for micro-organism, shortage of competent experts for the evaluation of the dossier and Company inexperience on the preparation of the dossier.

Problems are also related to the considerable amount of resources needed for the preparation of the Annex III dossier for the registration of product at National level, mainly related to the submission of a biological dossier requiring a number of trials as high as that of trials demanded for chemical products.

The sources for completing the data requirements are based on non protected studies, available scientific data from the open literature and proprietary GLP/GEP studies. Whenever information is missing, reasonable sensible justifications for not providing data has to be supplied. A free database with all the relevant literature information available should be created. Regarding the non-protected studies a list is not available, the Commission should generate it and notifiers should have access to it.

The taxonomic identification together with the characterisation of the micro-organism is the most important information in any risk assessment for a biotechnology product. Some uncertainties are related to the data requirements regarding the identity, mode of action and biology. The number of strains that notifiers have to use as reference for the identification of the active substance in the evaluation process is not defined. For the identification of the active substance required for the registration, the post-registration control and for monitoring purposes, the level of sensibility is not clearly defined. Validated tests are not available. A similar situation exists regarding the impurities (any component originating from the manufacturing process), and the metabolites, (products originating from degradative and biosynthetic reactions).

In relation to the persistence of the micro-organism in the environment no models are available. A proposal could be to develop appropriate FOCUS models for plant protection product based on micro-organism.

In the re-registration procedure another very important step is the definition of a task force agreement between notifiers of the same active substance. For the creation of a Consortium, at least six months are needed for the technical evaluation of the dossier and for finding an economical agreement. If the preparation of the dossier has to include studies referred to each single strain, it would be difficult or impossible to reach an agreement between notifiers. Usually each producer of biopesticides developed a plant protection product that is based on a proprietary unique strain. Effects of the genetic variability on the possible risk to human and animal health and the environment have to be evaluated by a group of experts. It is necessary that an uniform evaluation criterion be used by each one of the different Rapporteur Member States in the evaluation procedure.

Unfortunately, at present, the Annex VI to the Commission Directive 91/414/CEE with the uniform principle for the evaluation of micro-organism is still not available. The Commission encouraged the producers to submit collective dossiers in order to avoid duplication of work, and in particular experiments involving vertebrate animals. If the principle will be the submission of a dossier for each strain the Rapporteur member states might have to evaluate a number of dossiers for the same active substance. No doubt it will slow down the evaluation procedure and increase expense. In the event that only specific
studies would be referred to the strain it will be easier to create a task force between producers and to accelerate the evaluation procedure. It would be fundamental to address the question to a group of experts to consider the effects of the genetic variability between strains on the possible risk to human and animal health and the environment.

Commission on the draft document, Regulation laying down further details for the implementation of the fourth stage of the programme of work, define strict time-limits for all elements of the fourth stage of the work programme, in order to ensure its finalisation within an acceptable period of time. The deadline for the presentation of the dossier is set at the end of 2005. Assometab believes that the deadline Companies to submit the dossiers is too short and feels the need for special measures to be adopted in relation to the low risk compounds.
What will be the future for BCAs?
The industry’s point of view on problems in developing BCAs

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The discovery of a microorganism as a new potential candidate for biological control of a certain pest is the first of many steps in the development of a commercial biopesticide. The common thinking among scientists is that once a new Biocontrol agent (BCA) has been discovered, evaluated, some field and semi-field trials run and the study have been published, they do not see any further reasons to explain why the BCA has not been brought into actual use. Unfortunately, many other issues have to be taken into account (apart from registration).

First of all, I would like to stress the point that only small companies are really interested in the development of BCAs; in fact all cases in which a major company, dealing with conventional pesticides, ventured into the launch of a biopesticide resulted in total failure. Those companies do not have a correct approach to BCAs, the potential volume of business is normally too small for them and their staff lacks the mentality for handling successfully the production and the commercial distribution of biopesticides.

Apart from registration, three main issues have to be assessed to better understand commercial distribution of BCAs: 1) production, 2) quality control and 3) commercial distribution. Attaining the goal of producing a microbial pesticide at a cost affordable for the grower is very complex; the production process could be quite different from that of a “lab scale” unit, but it has to maintain the same standard both in quality and in yield. Not all companies have their own production facilities and consequently tolling manufacturing is common practice. Indeed, other problems like timing and production volume are limiting factors. In fact everybody understands that an increase of production volume would reduce production cost, but shelf life and storage issues of BCAs may become big obstacles. The result is that small companies prefer to produce lower volumes of biopesticides rather than running the risk of not selling enough product and suffering a decrease of product quality. As a consequence, costs for the end user are still high and in some periods of the year specific biopesticides might not be available.

Quality Control must be applied to every single product batch before distribution. Sometimes biopesticides carry on their label an expiry date, yet shelf life depends greatly upon storage conditions, not only at the dealer’s premises but even during transportation. Many a failure in the fields is due to the insufficient product quality, generated during the various steps of commercial distribution. Unfortunately, quality control procedures are generally applied only in the first phases of commercial distribution and very rarely at the end user’s level.

The distribution of a BCA has to be based on solid field application know-how; in practice, the BCA users have to be familiar with many technical details about biopesticides (e.g. spectrum of activity, target pests, timing of applications, compatibility with other pesticides, storage, shelf life, etc.) not to run the risk of failures. The information package should be disseminated among technicians and growers just before the commercial release of the product. Small companies are often unable to deliver the right information to all users, so mistakes in correct handling procedures are very common. But as soon as a new BCA is on the market the (small) producing company has to take a key decision: what kind of
distribution system to opt for. There are two potential alternatives: 1) direct contact with the grower; 2) conventional commercial channel; 3) “Cutting the middleman”, bypassing the conventional distribution system and establishing direct contact (and sales) to the growers is a tempting idea to the non-commercial people; a sort of dream for the biocontrol people, who normally realize that distribution channels are a monopoly of big chemical companies. This is partially true, but the distributors of BCAs have to take into account that bypassing the dealers (or the last ring of the distribution chain) will instantly mobilize dealers, distributing cooperatives and technicians of chemical companies against them (and their BCAs). Dealers, cooperatives and technicians will try and convince the grower that biocontrol does not work, that it is an ecologists and researchers dream, that pest damages occur suddenly in the field: in practice, the grower would run a big risk of losing his entire production just to replace a couple of “easy” and “cheaper” chemical treatments: a sort of psycho-agro-terrorism, normally very effective with the growers. This is the “best system” to guarantee failure; in fact at the first problem (since small companies cannot provide door-to-door field support) the grower will quickly go back to chemical treatments and he will never apply your BCA again. It has to be stressed that when a BCA fails biocontrol does not work “forever” and when a chemical does not work, there is always another, better one.

Using “conventional” distribution channels means that you will have to find some selected distributors as your potential ally, capable of assisting and helping the growers to face the occurrence of problems (take into account that dealers have a long experience in handling problems with chemical pesticides). In my experience, extension service people (especially when they are “public”) are quite reluctant to introduce novelties in the field of pests control, since even for them it is risky giving the advice to replace a chemical with a biopesticide without the pressure of problems (caused, for instance, by occurrence of resistance to chemicals, residues issues, toxicological matters, etc.). In fact, they are quite conservative and do not particularly wish to run the risk of affecting quality and quantity of the of the grower’s production only for the sake of replacing some chemicals. So BCAs would need support by someone really interested in their field development and knowledgeable in the problems of chemicals. To find dealers of this level is not easy. In fact the major part of them are not interested in distributing BCAs because of the shelf life/expiry date of the formulated products, of the need to store them at controlled temperature, of time to be spent for explaining and supporting the correct application of biopesticides and of lower margins for the BCAs sales (at least compared to the time spent “to sell” a chemical). Finally, the pressure by chemical companies (dealers are getting the major part of their income from chemical pesticides) could play an important role. Nevertheless, “conventional” distribution channels for agricultural products are the best system for BCAs and here lies the real challenge to enlarge the use of BCAs in agriculture. But some actions have to be taken into account. The dealers and the distributing cooperatives have to be carefully selected, since not all of them have the capability and the technical know how to handle biopesticides. The selection has to be based upon: 1) capability of providing a real field support; 2) facilities to store biopesticides; 3) know-how as to application of BCAs (compatibility with other pesticides, correct timing applications, knowledge of target pests, IPM strategy, etc.).

Finally, only few dealers can fulfill these demands, but this is the right way to ensure a profitable and durable commercial distribution to BCAs.

Conclusions

The commercial development of BCAs is delayed by some major constrains. Among these the need of storage, shorter shelf life (if compared with chemicals) and the consequent expiry
date, make the microbial biopesticides less attractive for commercial distribution. A key role has to be played by carefully selected good distributors to support in the field the BCAs application. It has even to be stressed that in theory the application of biopesticides in the frame of an IPM strategy, where the application of some chemical pesticides is integrated with the use of biopesticides, is assumed to be a very common practice. In the fields this is not true since extension service and growers do not introduce any novelties in the pest control system until they get problems like, for instance, the occurrence of resistance. In theory everybody realizes that the rotation of few biopesticides treatments with the majority of chemical treatments in a year-round strategy can easily reduce the risk of appearance of resistance without affecting the efficacy of the pest control. Unfortunately, this happens only very rarely and growers and technicians look for biocontrol only when chemical fails -and in some cases it is too late for a convenient BCAs application. This is a "residual" behaviour attributable to the "old style" pressure practiced by chemical companies in the sixties and seventies that carried to abuse of chemicals in agriculture; in fact growers and technicians often do not realize that the new active ingredients should be "protected" against the occurrence of resistance, since the cost of their development is very large and registration process of new molecules would be very long and expensive. The public extension service has to devote a big effort in trying to set up real IPM protocols forcing the inclusion of BCAs application. Another couple of constraints are the higher costs of BCAs (due mainly to the small-scale production) and the smooth, and sometimes hidden, competitive pressure by chemical companies onto dealers, growers and technical people. Indeed registration costs and the time needed for market penetration are very big issues for the development of biocontrol; only few companies can afford the cost of a registration at European level for products that by their very nature are targeted to a single or narrow spectrum of activity and necessarily have a "small" market.

Help may come by a more accurate control of chemical residues, the development of a (real) Organic Agriculture and some restriction in chemical usage (especially for the more toxic, cheap and old ones). Scientists and researchers can support the development of old and new BCAs by finalizing their researches to the field practices and not only in order to publish a study. Even the European Union has to act, by supporting not only basic research but even the development of procedures for the generation of registration files that have to become more flexible (and less expensive) for BCAs.