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Working group
“Multitrophic Interactions in Soil and Integrated Control”

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**Introduction**

The term trophic level is used to describe the feeding stratas of organism in the food web. In an agro-ecosystem one can identify many typical trophic groups that interact in standard food web nutrient cycle systems. However, other interactions at the trophic level occur that directly impact plant health and integrated management of pests and diseases. The trophic groups in the soil-ecosystem that are of primary interest to the IOBC Working Group – Multitrophic interactions in soil and integrated control - are manifold and include plant tissue consuming pathogens and pests and the multitude of microbial antagonists important in maintaining equilibrium in the system.

In many cases trophic group interactions are direct in the form of antagonists feeding directly on a pest or disease agent. In other cases they are more indirect, for example, interactions involving induced resistance or the production of toxic metabolites.

As demonstrated very clearly at this meeting, the number of interactions between trophic groups as they impact plant health are enormous and their uniqueness fathomless. The research papers presented at the IOBC Working Group meeting in Wageningen encompassed a broader spectrum of interactions than at any of the previous meetings. It goes without saying that "tunnel vision" was not on the agenda at this meeting.

The meeting was attended by scientists from 14 European countries and many graduate students from abroad. The papers presented came from the disciplines of mycology, bacteriology, nematology and entomology. For the first time a very strong contingent of soil ecologist attended the meeting. Their contributions expanded the horizon of all of those who attended as to how multitrophic interrelationships in the soil can be viewed and interpreted. Important was the fact that a large number of graduate students attended and actively participated in the meeting. They added significantly to the overall outcome of the meeting and we hope they continue to interact with us.

The majority of the papers presented at the meeting and many of the poster presentations have been compiled by Jos M. Raaijmakers in this bulletin, the result of which is a “good read” for anyone interested in soil ecosystems and integrated control. We hope this leads to increased interest in the wonderful world of multitrophic interactions in the soil and integrated control. We hope to see everyone at the next meeting in France in 2007.

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Active role of microbial life in soil suppressiveness to *Rhizoctonia solani*

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**Abstract:** *Rhizoctonia solani* anastomosis group 2-2IIIB causes damping-off, black root rot and crown rot in sugar beet (*Beta vulgaris*). Based on experiences of growers and field experiments, soils can become suppressive to *R. solani*. The fungus may be present in the soil, but the plant does not show symptoms. Understanding the mechanisms causing soil suppressiveness to *R. solani* is essential for the development of environmentally friendly control strategies of rhizoctonia root rot in sugar beet. A bioassay that discriminates soils in their level of disease suppressiveness was developed. Results of bioassays were in accordance with field observations. Irradiation and heating of soil samples eliminated soil suppressiveness, indicating an active role of microbial life in soil suppressiveness to *R. solani*. Furthermore, soil suppressiveness seemed to be built up in presence of *R. solani* that may indicate the involvement of hyperparasites in soil suppressiveness. Preliminary results suggest that for development of soil suppressiveness to *R. solani*, soil microbial life and *R. solani* are important components and to a lesser degree the host. Our research focuses on hyperparasites through bioassays (Cambridge Method), *in vitro* techniques and molecular techniques (PCR-DGGE).

**Keywords:** disease suppression, *Beta vulgaris*, Cambridge Method, hyperparasites

**Introduction**

*Rhizoctonia solani* anastomosis group (AG) 2-2IIIB causes damping-off, black root rot and crown rot in sugar beet. The fungus is typically a facultative parasite that can survive saprophytically for long periods, almost exclusively in the form of sterile mycelium associated with organic debris in soil (Blair, 1942; Boosalis and Scharen, 1959). *R. solani* AG 2-2IIIB has a wide host range such as maize, lily, gladiolus, carrot and beans. The disease occurs in patches which can differ in time and space. Management of rhizoctonia includes the use of rhizoctonia resistant sugar beet cultivars, providing a good soil structure, weed control and crop rotation with non-hosts. The resistance of rhizoctonia resistant sugar beet varieties is, however, partial, implying that the degree of resistance is influenced by the inoculum potential of *R. solani* and by environmental factors. Furthermore, seedlings are still susceptible for rhizoctonia. In addition to the choice of resistant varieties and good farming practices other cultural practices are needed to manage rhizoctonia.

Soils suppressive to *R. solani* in sugar beet have been observed in The Netherlands (Anonymous, 2001) and in Japan (Hyakumachi and Ui, 1982; Hyakumachi et al., 1990). A disease suppressive soil can be defined as a soil in which disease development is suppressed even though the pathogen is introduced in the presence of a susceptible host (Baker and Cook, 1974). Understanding the mechanisms causing soil suppressiveness to *R. solani* is essential for the development of environmentally friendly control strategies of rhizoctonia root rot in sugar beet.

Disease suppression that was observed in the field could be simulated in a bioassay (Bakker and Schneider, 2004). The bioassay discriminates soils in their level of disease
suppressiveness and facilitates the study to the causal mechanisms of disease suppression. This paper deals with the active role of microbial life in disease suppression and the possible involvement of hyperparasites through bioassays (Cambridge Method), in vitro techniques (Antagonism experiment) and molecular techniques (PCR-DGGE).

**Materials and methods**

**Active role of microbial life**
Two disease suppressive soil samples and one disease conducive soil sample were gamma-irradiated to destroy microbial life completely or were heated either at 50°C or 80°C to destroy microbial life partially. After irradiation or heating, soil samples were artificially infested with a three-weeks-old culture of *R. solani* AG 2-2IIB. The culture was a mixture of fine river sand (0-1 mm; pH 6-6.5; 0% organic matter) with pasteurised potting soil (9:1 w/w) and oat meal (3.4% w/w) that was inoculated with mycelial plugs of *R. solani*. Soil was put into PVC tubes (Ø 2 cm, height of 15 cm) and sugar beet seeds were directly sown. The bioassay comprised 12 replicates and was performed in a climate room at 23°C during the day, 15°C during the night, a photoperiod of 14 h and a relative humidity of 95%. Disease assessment was after four weeks of sowing on a scale from 0 (seedling healthy) till 3 (seedling dead).

**Effect of soil storage on soil suppressiveness**
A disease suppressive soil sample was collected in 2003, air-dried for one week and was stored at 23°C for one year. Disease index of this soil sample was determined in 2003 and after one year of storage.

**Cambridge Method**
The Cambridge Method according to Garrett (1970) was modified. The competitive saprophytic ability (CSA) of *R. solani* was determined in both a disease-conducive soil and two disease-suppressive soils. The hypothesis was that the CSA of *R. solani* is lower in disease-suppressive soils than in disease-conducive soil because of the presence of antagonistic microorganisms in disease-suppressive soils. Different inoculum densities of *R. solani* (0, 0.1, 1, 10 and 50% w/w of three-weeks-old oat meal culture infested with *R. solani*) were mixed together with 10 dead sugar beet seeds in the soil. PVC tubes (Ø 2 cm and height of 15 cm) were filled with soils and soils were moistened to about field capacity. During the assay the soils were watered daily. The experiment comprised 6 replicates per treatment and was performed in the climate room at 23°C. After four weeks of incubation the seeds were retrieved from the soil and rinsed with tap water. Seeds were placed on a semi selective medium with antibiotics (50 mg/L streptomycin and 50 mg/L chloroamphenicol) and after 24 h the number of seeds colonized by *R. solani* was determined by the microscope. Soil samples were collected for PCR-DGGE analysis to determine which antagonistic microorganisms may be involved in soil suppressiveness. Soil samples were also collected for bioassays to determine the infection potential of *R. solani*.

**Antagonism experiment**
The hypothesis was that growth of hyperparasitic fungi is stimulated at a high inoculum density of *R. solani*. Therefore, soil infested with 50% (w/w) of three-weeks-old oat meal culture of *R. solani* from the modified Cambridge Method was spread over one-week-old potato dextrose agar plates that were overgrown with *R. solani*. The outgrowth of fungi was screened for hyperparasitism on the hyphae of *R. solani*. 
PCR-DGGE
A large fraction of the soil microbial community can not be cultivated on growth media, hence, the molecular tool, PCR-DGGE, was used instead. Total genomic DNA was extracted from the soil samples of the modified Cambridge Method. 18S rDNA was amplified with generic fungal primers (Vainio et al., 2000). Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode gel system (BioRad).

Results and discussion

Active role of microbial life
Disease index was for both suppressive soil samples significantly higher after irradiation and after heating at 80°C than when suppressive soil samples were not treated (Table 1). These results indicate an active role of microbial life in disease suppression.

Table 1. Mean disease index of disease suppressive soil samples (SS) and a disease conducive soil sample (CS) that have received four treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SS 1</th>
<th>SS 2</th>
<th>CS 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>no</td>
<td>0.6</td>
<td>0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>irradiation</td>
<td>1.8</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>50°C</td>
<td>1.4</td>
<td>1.3</td>
<td>2.5</td>
</tr>
<tr>
<td>80°C</td>
<td>2.1</td>
<td>2.4</td>
<td>3.0</td>
</tr>
</tbody>
</table>

1 Mean disease index from 0 (seedling healthy) till 3 (seedling dead). LSD 5% = 0.9 (REML, Genstat)

Effect of soil storage on soil suppressiveness
Disease index of a disease suppressive soil sample was determined directly after soil collection (2003) and after one year of storage (2004). Disease index was significantly increased (LSD 5% = 0.6) from 0.4 in 2003 to 2.3 in 2004. Disease index was significantly decreased from 2.3 to 0.5 after addition of mycelium of R. solani to this soil sample. So soil suppressiveness was reduced after storage, but could be induced after extra addition of mycelium of R. solani. Microbial life seemed to be built up in the presence of R. solani. Addition of live mycelium of R. solani to soil markedly stimulated the growth of hyper-parasitic fungi according to Jager et al. (1979) and Van den Boogert and Jager (1983).

Cambridge Method
The competitive saprophytic ability of R. solani was lower in disease suppressive soils compared to its competitive saprophytic ability in disease conducive soil (Figure 1). Antagonistic microorganisms may be present in disease suppressive soils that hamper the colonization of substrates by R. solani. In disease suppressive soil, R. solani was not able to colonize substrates and to infect sugar beet seedlings. These results suggest that R. solani may have been parasitised by microorganisms. In that case, the inoculum potential of R. solani, the energy that is needed for establishment, survival, growth and infection, will be reduced (Garrett, 1970). In disease conducive soil, R. solani was able to colonise substrates, but was not able to infect sugar beet seedlings. Colonization of substrates as a food resource may cost a lot of energy. Consequently, the pathogen may save its energy when plant roots are available. Furthermore, for the development of soil suppressiveness to R. solani, soil microbial life and R. solani seemed to be important components and to a lesser degree the host.
Figure 1. Percentage of dead sugar beet seeds colonized by *Rhizoctonia solani* in a disease-conducive soil (CS) and in two disease-suppressive soils (SS) infested with different inoculum densities of *R. solani* after four weeks of incubation at 23°C. Inoculum was made of a mixture of fine river sand (0-1 mm; pH 6-6.5; 0% organic matter) with pasteurised potting soil (9:1 w/w) and oat meal (3.4% w/w) that was inoculated with mycelial plugs of *R. solani*. Inoculum density of 50% means that half of the total soil comprised inoculum.

**Antagonism experiment**

*T. harzianum* was associated with soil suppressiveness to *R. solani* in radish in pot experiments. Other mycoparasites of *R. solani* that have been described are *Gliocladium virens* (Tu and Vaartaja, 1981), *Pythium oligandrum* (Al-Hamdani and Cooke, 1983), *Verticillium biguttatum* (Van den Boogert and Deacon, 1994) and *Chaetomium spirale* ND35 (Gao et al., 2005).

**PCR-DGGE**

Soil samples that were collected from the Cambridge Method were analyzed with PCR-DGGE. Fungal fingerprint of the disease conducive soil was compared to the fungal fingerprints of disease suppressive soils. Variation in fungal fingerprints between disease conducive soil and disease suppressive soils was observed. We are especially interested in hyperparasites that are present after addition of high inoculum density of *R. solani* to disease suppressive soil.
References


Biocontrol of *Rhizoctonia solani* on cucumber seedlings by *Trichoderma* spp.

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Abstract: Rhizoctonia damping-off in seedlings is one of the most important sanitary problems in a wide range of plant species in Brazil. Species of *Trichoderma* have been shown to be particularly effective to control soil-borne plant pathogens because they can grow very fast in different soils and substrates and use a diverse antagonistic mechanisms to “exclude” the pathogens. In greenhouse assays, 250 *Trichoderma* spp. isolates were evaluated as biological control agents of *Rhizoctonia solani* AG-4 on cucumber seedlings. The results demonstrated that forty seven isolates reduced the cucumber post-damping-off disease from 10 to 100%, and 22 were promising as biological control agents of the pathogen.

Introduction

Damping-off is an important disease of a wide range of seedlings in nurseries, glasshouses, gardens, crops, and forests (Agrios, 1997). Among the most common pathogens responsible for this disease are different species of *Pythium* and several anastomosis groups (AG) of *Rhizoctonia solani* (Lumsden & Locke, 1989). *Rhizoctonia solani* is a ubiquitous soilborne plant pathogen with worldwide distribution and can cause pre- and post-emergence damping-off and root rot in many crops (Anderson, 1982; Dorrance et al., 2003). Increased societal concerns related to the use of agrichemicals and genetically modified organisms as means of managing crop diseases has prompted greater interest in biological control (Bargabus et al., 2004). *Trichoderma* spp. are well documented as effective biological control agents of plant diseases caused by soilborne fungi (Papavizas, 1985, Melo, 1991; Howel, 2003). The objective of this paper was to evaluate the capacity of 250 *Trichoderma* spp. isolates to control Rhizoctonia damping-off disease in cucumber seedlings.

Material and methods

*Trichoderma* spp. isolates were obtained from 65 soil samples collected all around São Paulo state in Brazil, from conventional and organic farming systems properties and in preserved native vegetation areas of agricultural lands. *Rhizoctonia solani* AG-4 was isolated from diseased cucumber plant from Campinas-SP. Two hundred and fifty *Trichoderma* spp. isolates were screened in five greenhouse tests to select the potential biocontrol agents against Rhizoctonia post-emergence damping-off disease. Commercial nursery substrate (Plantmax/Eucatex Agro) infestation was performed by adding 20g of colonized wheat grains with pathogen and 20g of rice grains with *Trichoderma* sp. isolate (w/v grains/substrate) per liter. This mixture was homogenized and incubated for seven days at room temperature. Ten milliliters of the mixture were applied near the basal part of two weeks old cucumber plants and the number of surviving and collapsed seedlings was counted seven days after. The assays were arranged in completely randomized designs. Each treatment contained four replicates, with five plants per pot. Analyses of data were done by Tukey’s test.
Results and discussion

Among the 250 Trichoderma spp. isolates tested, forty seven isolates (19%) reduced the disease incidence from 10 to 100%. The best 22 antagonistic isolates were significantly effective (P=0.05) against Rhizoctonia post-emergence damping-off, compared with the pathogen control treatment, and six of them (IB32/04, IB34/03, IB45/15, IB48/03, IB49/08 e IB49/17) reduced the disease incidence 100% (Figure 1).

The results showed that a good percentage of tested Trichoderma spp. isolates, 19%, acted as a strong barrier for the spread of R. solani in the commercial nursery substrate, once they were applied at the same time and in the same amount as the pathogen. This indicates that competition might be associated with this control. Species of Trichoderma have multiple mechanisms of action, including mycoparasitism, antibiosis, competition and induced resistance (Howell, 2002). According to Lumsden and Lock (1989) a short period of incubation of the pathogen with the antagonist to reach the control of Rhizoctonia damping-off disease is required. In our assays, the mixture prepared with pathogen and antagonist was incubated for one week before it was applied to the cucumber seedlings, indicating that this period was enough to allow the pathogen control. Although 22 isolates significantly reduced the disease incidence, other 25 isolates also showed some controlling effect, superior to 10%, against the pathogen, indicating that further research is needed to define the best conditions in which each isolate can be used effectively. The approach of introducing the biocontrol agent into the nursery substrate is an excellent application of a biological control measure to reduce or prevent losses caused by damping-off pathogens, once the protection from damping-off diseases is most critical during the early stages of seedling development. However, optimal application of the promising selected isolates will have to wait further evaluation and research to elucidate the isolates modes of action.

Figure 1. Effect of Trichoderma spp. isolates on cucumber damping-off caused by Rhizoctonia solani in potting medium. Bar values with the same letter do not differ according to Tukey’s test (P=0,05) (CR= Rhizoctonia control and CB= blanc control).

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References

A potential role for collagen in the attachment of *Pasteuria penetrans* to nematode cuticle

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Abstract: The Gram-positive bacterium *Pasteuria penetrans* is a parasite of root-knot nematodes *Meloidogyne* spp. and has potential as a biological control agent. The bacterium produces endospores that adhere to the cuticle of second-stage juveniles as they migrate through the soil. Immunological heterogeneity of the surface of the endospore is related to host specificity. Recent research has shown the surface of *Bacillus anthracis* contains protein filaments made up of a collagen-like glycoprotein (BclA) the gene of which flanks the rhamnose biosynthesis operon; the length of these filaments in different strains of *B. anthracis* has been shown to be determined by the number of collagen-like (G-x-y) repeats. Similar genes are also present in *B. cereus* and *B. thuringiensis*. A genome sequencing project of *P. penetrans*, has produced 4000 unique sequences and a database of these sequences has been screened for BclA using BLAST. Several collagen-like proteins have been identified in *P. penetrans* that contain G-x-y repeats and these sequences appear to be closely related to those of *Bacillus thuringiensis*. The pre-treatment of endospores with either collagenase or the collagen binding domain of fibronectin inhibited endospore binding to nematode cuticle suggesting that collagen-like proteins are also present on the exosporium surface of *P. Penetrans* and involved in attachment.

Key words: Biological control, *Bacillus thuringiensis*, *Bacillus anthracis*, BclA, endospore *Meloidogyne*, Collagen

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are a major pest of economic importance in world agriculture. These nematodes are classified as sedentary parasites, because, after migrating through the soil and invading a root, each has to set up a specialised feeding cell and can then no longer move. During the development of the feeding cell and the growth of the nematode the plant roots develop galls and become distorted, a process which affects the ability of the roots to function. *Pasteuria penetrans* is a Gram-positive hyperparasitic bacterium and all the economically important plant parasitic nematode pests have been observed, at some stage, either to be encumbered with endospores of the bacterium adhering to the nematode cuticle, or to have been infected with the bacterium and endospores seen inside the body cavity (Chen and Dickson, 1998). There are two principal reasons that limit the bacterium being developed as a commercial biological control agent; a) the inability to mass produce the organism *in vitro*, and b) host specificity, one population of endospores will adhere to and infect one population of nematode but not another (Davies *et al.*, 2001). Recent work at Pasteuria Biosciences LLC, Alusha, Florida has made considerable progress in the development of an *in vitro* production system for *Pasteuria*, but little research has focused on the problem of host specificity. This manuscript aims at reviewing some of the research undertaken on understanding the mechanism of endospore attachment and presents some recent research findings from a survey of the *Pasteuria* genome that sheds light onto the molecules on the surface of *Pasteuria* spores that may be important in the attachment process.
Materials and methods

Immunoology and host specificity
Monoclonal antibodies raised to whole endospores of *P. penetrans* were produced in Balb/C mice using standard methods and screened for their ability to recognise the surface of the endospores by immunofluorescence (Davies et al., 1994). The endospores used in the immunisation were produced on infected *M. incognita* Race 2. Five monoclonal antibodies were produced but they did not all recognise the endospores equally. Monoclonal antibodies Mab-12, 53, 117 and 134 recognised 30, 25, 21 and 28 percent of endospores respectively while Mab-84 only recognised 3 percent of endospores. This result shows that the population of endospores produced from a single egg mass population was heterogeneous. However, in a baiting experiment where different nematode populations were incubated in a standardised concentration of endospores and the spores adhering to the nematode cuticle characterised by using the monoclonal antibodies, it was shown that different sub-populations of the endospores adhered to the different nematode populations (Figure 1)

![Figure 1](image)

This result shows that not only is there heterogeneity present on the surface of the endospores but that there must also be heterogeneity present on the nematode cuticle.

*Pasteuria* genome sequencing and collagen
Four genomic libraries from a population of *P. penetrans* strain RES147, which has a broad host-range, have been produced using a whole shotgun sequencing approach. This has produced some 9074 sequence reads that have resulted in 2.8 Mbp of primary, quality trimmed genomic sequence (Bird et al., 2003). Approximately 50% of the sequence has yielded significant (e-value < 1.0e-10) similarities and significant co-linearity has been observed between *P. penetrans* and
Bacillus subtilis (Bird et al., 2003). Using 27 housekeeping genes that have been identified in Pasteuria that are also present in a whole range of Gram positive and Gram negative bacteria it has been possible to construct a phylogenetic tree and the results suggest that Pasteuria may be ancestral to other Bacilli such as B. subtilis, B. halodurans, that are saprophytes, and B. thuringiensis and B. anthracis that are pathogens (Charles et al., 2005).

Endospores are highly resistant spores containing a central body surrounded by a spore coat that, in turn, is surrounded by another prominent layer called the exosporium. Studies have revealed that the exosporium of pathogenic Bacilli, (B. thuringiensis, B. cereus and B. anthracis) contained a paracrystalline basal layer and a hair-like nap. This hair-like nap is not present on non-pathogenic Bacilli (eg. B. subtilis). Pasteuria penetrans also produces endospores and although they are morphologically distinct with much greater morphological differentiation when compared to other Bacilli, its exosporium also contains a hair-like nap that is in intimate contact with the nematode cuticle on endospore attachment (Persidis et al., 1991). Recent work with Bacillus anthracis has attempted to characterise this hair-like nap and it has been shown to be a collagen-like glycoprotein called BclA (for Bacillus collagen-like protein of anthracis). BclA is the structural component of the hair-like nap and contains a number of G-x-y collagen-like repeats (Sylvestre et al., 2003). The lengths of the filaments composing the nap of different strains of B anthracis differ and the number of G-x-y repeats is responsible for the length of the filaments on different strains of the bacterium (Sylvestre et al., 2003). A regression analysis of the relationship between both the number of G-x-y triplet repeats shows it to be highly significant (Figure 2).

![Figure 2. Regression between number of G-x-y triplet repeats in BclA from Bacillus anthracis and filament length calculated from Sylvestre et al., 2003](image)

Research from a Pasteuria genome sequencing project has revealed a number of sequences that code for the collagen-like protein sequences and using the equation derived from the regression of the number of G-x-y triplets against filament it is possible to make a prediction of the filament lengths in P. penetrans (Table 1).
Table 1. Three contigs from the *P. penetrans* sequence survey showing the number of G-x-y triplet repeats, the number of amino acids this represents and the expected collagen filament length (calculated from the equation \( y = 3.1428\times - 31.182 \) from Fig 2), and the results of a BLASTp comparison between *Pasteuria* contigs and those of *Bacillus anthracis*, *B. cereus* and *B. thuringiensis*

<table>
<thead>
<tr>
<th>Pasteuria contigs</th>
<th>G-x-y</th>
<th>Amino acids</th>
<th>Filament length (nm)</th>
<th>BLASTp: % similarity (E-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c178</td>
<td>78</td>
<td>234</td>
<td>213.9</td>
<td>52 (5e-67) 52 (3e-66) 52 (2e-67)</td>
</tr>
<tr>
<td>c280</td>
<td>28</td>
<td>84</td>
<td>56.8</td>
<td>48 (3e-15) 50 (4e-14) 42 (2e-13)</td>
</tr>
<tr>
<td>c374</td>
<td>35</td>
<td>105</td>
<td>78.8</td>
<td>60 (2e-29) 64 (6e-33) 63 (1e-30)</td>
</tr>
</tbody>
</table>

Discussion

The survey of the *P. penetrans* genome has been useful in identifying several putative collagen-like proteins present in *Pasteuria*. Based on the BLASTp results contig c178 is most closely related to *B. thuringiensis* followed by *B. cereus* and least related to *B. anthracis* with all these three bacteria having a 50 % similarity and with E-values < 3e\(^{-66}\). Contig c374 showed the next greatest level of similarity with E-values ranging from between < 2e\(^{-38}\) to 6e\(^{-33}\) with *B. anthracis* being the least similar. Contig c280 showed the least similarity to any of the *Bacillus* species tested and showed greater similarity to collagen-like proteins occurring in *Streptomyces coelicolor* (54 % similarity with E-value 1e\(^{-18}\)). These results therefore show that several species of collagen-like protein occur in *Pasteuria* and in general they are more closely related to *B. thuringiensis* and *B. cereus* than *B. anthracis*. The question therefore to be adressed is are these collagens present on the surface of the endospore. There a two main pieces of evidence that suggest collagen is present on the surface of the endospore; 1) earlier research has shown that the pre-treatment of endospores with collagenase reduces the ability of endospores to attach to nematodes (Davies et al., 1993) and further research done at Rothamsted has shown that endospores pre-incubated with the collagen-binding domain of fibronectin also prevented endospores of *Pasteuria* attaching to the nematode cuticle (Mohan et al., 2001). This work suggests that a collagen-like molecule is present on the surface of the endospore and therefore may be responsible for the attachment of endospores but whether or not collagen is sufficient to account for the attachment specificity observed between different populations of *Pasteuria* and different nematode populations remains to be seen; different lengths of the collagen-like filaments may account for the variation observed in endospore attachment.

Acknowledgements

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References


Role of iron-regulated metabolites in *Arabidopsis* root colonization by *Pseudomonas fluorescens* WCS374

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Abstract: The plant growth-promoting rhizobacterium *Pseudomonas fluorescens* WCS374 produces several iron-regulated metabolites, including the fluorescent siderophore pseudobactin, salicylic acid (SA), and pseudomonine, a siderophore that contains a SA moiety. To study the functional role of pseudomonine and SA in the colonization of roots of *Arabidopsis thaliana*, genes *pmsA* and *pmsB* of the pseudomonine biosynthesis gene cluster were disrupted using homologous recombination. These mutants, defective in SA and pseudomonine biosynthesis, were further subjected to Tn5 mutagenesis to generate mutants also deficient in pseudobactin production. The resulting double mutants still appeared to have siderophore activity. The SA/pseudomonine and the double mutants colonized *Arabidopsis* roots to the same extent as the wild type bacterium. Surprisingly, a mutant that produced SA and pseudomonine but lacked pseudobactin colonized the plant roots significantly better. Under the conditions tested production of iron-regulated metabolites does not seem necessary for effective colonization of the *Arabidopsis* rhizosphere by WCS374.

Key words: *Pseudomonas fluorescens*, Pseudobactin, Root colonization, Salicylic acid, Siderophore

Introduction

Under conditions of iron limitation, microorganisms produce siderophores that sequester iron from the environment and subsequently transport it into the microbial cell. When iron becomes limited, the rhizobacterium *Pseudomonas fluorescens* strain WCS374 produces the fluorescent siderophore pseudobactin, salicylic acid (SA), and pseudomonine, a siderophore that contains a SA moiety (Mercado-Blanco et al., 2001). Strain WCS374 can induce systemic resistance in radish (Leeman et al., 1995) but not in *Arabidopsis thaliana* (Van Wees et al., 1997), whereas exogenously applied SA induces resistance in both plant species. Compared to other bacteria that have been described to produce SA, WCS374 produces high amounts *in vitro* (Leeman et al., 1996), but it is not clear if it releases SA in the rhizosphere or not. Since application of exogenous SA induces resistance, the inability of WCS374 to induce resistance in *Arabidopsis* suggests that SA is not released into the rhizosphere. Instead, it might all be channeled into pseudomonine. Therefore, a study was undertaken to determine the conditions in which SA and pseudomonine are produced in the rhizosphere.

Effective root colonization by *Pseudomonas* spp. is a prerequisite for successful suppression of soil-borne plant diseases. A minimum bacterial cell density is required for both microbial antagonism and the induction of systemic resistance (Raaijmakers et al., 1995). Moreover, time is required for the bacterium to express its disease-suppressive properties. Specific mutants of *P. fluorescens* WCS374 were generated to study the role of iron-regulated metabolites in the ecology of the bacterium and its ability to induce systemic resistance. Here, we report observations on the importance of these metabolites for the ability of WCS374 to colonize *Arabidopsis* roots.
Materials and methods

**Bacterial strains, growth conditions and determination of SA**

*P. fluorescens* WCS374 was originally isolated from the rhizosphere of potato (Geels and Schippers, 1983). A Tn\(^5\) insertion mutant of this strain that lacks pseudobactin production was described by Weisbeek *et al.* (1986). For the colonization studies strains were grown on King’s medium B agar plates for 24 h at 28°C. Bacterial cells were scraped off the plates and suspended in 10 mM MgSO\(_4\). For determination of SA production WCS374 was grown in liquid standard succinate medium (SSM) for 48 h. Culture supernatants were extracted with chloroform as described before (Mercado-Blanco *et al.*, 2001). SA was quantified by measuring the absorbance of the deep purple Fe\(^{3+}\)-SA complex at 527 nm.

**Arrangement of pseudomonine biosynthesis and transport genes in WCS374**

An EcoRI fragment cloned in plasmids pE1R and pE4R that contains 15.795 bp of the 28-kb fragment from WCS374 responsible for SA and pseudomonine biosynthesis and transport was sequenced following a primer-walking strategy. Sequencing was performed in both directions. Sequences were edited using DNASTAR (Lasergene) software. Homology analysis was done with the BLAST program at the NCBI network service.

**SA biosynthetic mutants of WCS374**

We employed homologous recombination to knock out either gene *pmsA*, which codes for histidine decarboxylase, or gene *pmsB*, which codes for isochorismate-pyruvate lyase. A kanamycin resistance cassette from plasmid pUC4K was introduced within these genes and the disrupted genes were cloned in the suicide plasmid pSUP202. The recombinant plasmids were used for homologous recombination with WCS374.

**Mutants defective in the production of pseudobactin**

The *pmsA* and *pmsB* deficient mutants were subjected to Tn\(^5\) mutagenesis to isolate mutants additionally defective in pseudobactin production. The mobilization system of *Escherichia coli* strain S17-1 and the suicide plasmid pJQ18, which carries a modified Tn\(^5\) (Mob Tc\(^3\)), were used.

**Determination of iron-chelating capacity**

Iron-chelating capacity of the bacteria was quantified by relative halo size around colonies grown on Chrome Azurol S (CAS) medium, which is a universal medium for the determination of siderophore activity. Production of siderophore leads to a shift in colour from blue (chelated CAS) to orange (free CAS).

**Colonization of Arabidopsis roots by WCS374 and its siderophore mutants**

Seedlings of *A. thaliana* Col-0 were grown in sterile sand for two weeks. Three plantlets were transplanted into 60-ml pots containing a mixture of sand and potting soil that was autoclaved twice for 1 h with a one-day interval. Before transferring the plantlets, the potting soil was supplemented with a suspension of bacteria to a final density of 10\(^7\) cfu/gram soil. Plants were watered on alternate days and once a week supplied with a modified half-strength Hoagland nutrient solution without iron. Roots were sampled and the numbers of bacteria per gram of root were determined by plating serial dilutions of rhizosphere suspensions in 0.01M MgSO\(_4\) on media with appropriate antibiotics one, two and three weeks after transplanting.

Results and discussion

**SA and pseudomonine biosynthesis genes in WCS374**

The SA and pseudomonine biosynthesis genes in WCS374 are organized in a cluster designated *pmsCEAB* (Figure 1A), containing two ORFs (*pmsB* and *C*) with homology to chorismate-utilizing enzymes, one (*pmsE*) with homology to enzymes involved in siderophore
biosynthesis, and one \((\text{pms}A)\) encoding a putative histidine decarboxylase (Mercado-Blanco et al., 2001).

Additional ORFs involved in pseudomonine biosynthesis and transport have now been identified (Figure 1B). Three ORFs (1-3) may complete the pseudomonine biosynthesis machinery, together with the previously characterized \(\text{pms}CEAB\) genes. Additional ORFs (5–10) were identified that may be involved in transport and uptake of pseudomonine.

**Figure 1.** Salicylic acid (A) and pseudomonine biosynthesis and transport coding region (B) in *Pseudomonas fluorescens* WCS374.

### Salicylate and double mutants

Kanamycin (Km)-resistant, tetracyclin (Tc)-sensitive colonies were selected in a two-step screening procedure. Presence of a unique insert within the disrupted genes was confirmed using primer pairs SAL01/SAL02 and SAL02/HDC01, which amplified the \(\text{pms}B\) and \(\text{pms}AB\) regions of the genome (Mercado-Blanco et al., 2001), respectively. The mutants no longer produced SA in iron-limiting liquid SSM, as determined by chloroform extraction. Tc-resistant colonies were obtained after conjugation of the SA/pseudomonine mutants with *E. coli* S17-1 carrying the Tn5 transposon. Colonies that were non-fluorescent on King’s medium B, indicating that pseudobactin production was abolished, were selected. These double mutants still had siderophore activity as measured on CAS medium. This observation suggests the presence of yet another molecule with siderophore activity in WCS374.

### Colonization of Arabidopsis roots by WCS374 and its mutants

All mutants colonized *Arabidopsis* roots to levels similar to the wild type. Surprisingly mutants that lack pseudobactin but still produce SA and pseudomonine, reached up to 10-fold higher cell densities. Population densities of WCS374, SA and double mutants increased to...
about 10^6 cfu/g root within the first week after transplanting the seedlings. Thus, iron-regulated metabolites do not seem to contribute to effective colonization of the Arabidopsis rhizosphere by WCS374. Pseudobactin production even seems to reduce root colonizing capacity. The mutants are currently used to investigate the role of the iron-regulated metabolites of WCS374 in the induction of systemic resistance in Arabidopsis and radish.

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References


Trichoderma and soil solarization induced microbial changes on plant surfaces

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Abstract: Both soil solarization and Trichoderma reduce disease incidence. In one work we studied the effect of both treatments on rhizosphere populations and in a second work we studied the effect of Trichoderma on phyllosphere populations. In both works the effect of the treatments on foliar pathogens was studied. When the roots were in contact with the solarized soil or Trichoderma and the foliage was inoculated with pathogens, strawberry, cucumber and common bean grown on treated media showed significant reduction in disease after leaf inoculation with Botrytis cinerea or with Sphaerotheca fuliginea, hence indicating induced resistance. Attempts were made to find the relationship between indigenous populations of microorganisms in the rhizosphere or in the phyllosphere and disease control. For this purpose we used a molecular approach based on 16S-rDNA and denaturizing gradient gel electrophoresis (DGGE). Bacterial 16S ribosomal DNA (rDNA) extraction from roots and leaves that were subjected to the various treatments was PCR amplified with chosen primers. Amplicons were separated by size and base composition by DGGE in order to fingerprint shifts in the structure of the natural plant-associated microbial communities that may result from the treatments. The soil treatments resulted in changes in the DGGE patterns of rhizosphere and phyllosphere populations. Some bacilli, pseudomonads and actinobacteria were detected and their role in induced resistance is currently tested. T. harzianum treatment to leaves or roots resulted in increased variability in the bacterial population inhabiting the leaves. It is suggested that some of the effect exerted by soil solarization and Trichoderma are associated with microbial changes.

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

Introduction

Plants are frequently exposed to a variety of pathogens, which all have to be controlled. Integration of methods for disease management, if appropriately applied, results in improved and a wider spectrum of disease control, while reducing chemical use. Research on microbial interactions in agricultural ecosystems has yielded a vast amount of knowledge about alternative control of plant pathogens. Infection by pathogens can be reduced by prior inoculation of the plant surfaces with biocontrol agents to the soil or canopy or by treating the soil with solarization. Microbial biocontrol agents (BCAs) and soil solarization 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.

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 (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 antagonistic to many pathogens. *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). Among the first *Trichoderma* biocontrol products which were developed and commercialized worldwide is the preparation TRICHODEX, that contains isolate T39 of the disease-antagonistic fungus *T. harzianum*. *T. harzianum* T39 controls *B. cinerea*, *S. sclerotiorum* and several other plant pathogens on grape vines, greenhouse crops and orchards (Elad, 2000; O’Neill, 1996).

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 less attention.

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 (Kloeper et al., 1992; Kuć, 2002). 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 mechanism of resistance induced by *Trichoderma* was recently demonstrated in cucumber (Shoresh et al., 2005). The objective of our works was to study the effect of soil solarization and *Trichoderma* application to soil, on plant growth, induced resistance and microbial populations.

**Materials and methods**

**Treatments and experimental growing conditions**

In one study, conidia suspension of the fungus *T. harzianum* T39 (10⁶/ml) and its powder formulation were either applied to the same leaf as the pathogen or to the roots. *B. cinerea* infection was carried out with conidial suspensions (10⁵/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.

In a second work we tested the effect of soil solarization and *Trichoderma* for integrated control. Three treatments were carried out in sandy loam soil, 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 (3 g/L) to the soil. 3. combination of solarization and *T. harzianum* T39. Cucumber and common bean seeds were sown in the treated soils. 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 calculated. Inoculation with the cucumber powdery mildew pathogen *Sphaerotheca 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.

**Denaturating 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, Bacillus* and *Actinobacteria* then loaded and run on the DGGE according to Muyzer et al. (1993). Dendrograms of the different lanes presented in the GDDE gels were created.

**Results and discussion**

**Microbial populations**

We used PCR-DGGE for assessing microbial changes in the rhizosphere. 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 and formamide 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 denaturants. This means that cDNA from 16S 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 databases can then provide a classification for all the species in a particular niche.

In one work we studied bean leaves for multi-level interactions of the BCA *T. harzianum* T39, the plant pathogen *B. cinerea* and indigenous microflora. DGGE 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 root zone or to the leaves 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 direct application to the leaves or indirect application to the roots. Indeed, representative isolates from the phyllosphere antagonized *Botrytis in vitro* and on bean leaves following canopy treatment (Elad et al., 2004). 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.

DGGE-PCR analysis was also performed in a second study involving *T. harzianum* applied to soil and soil solarization. When using universal primers for bacteria, different band patterns were obtained from the rhizosphere of plants grown on untreated soil compared to plants grown on solarized and TRICHODEX treated soil (Figure 1). Specific primers for pseudomonads, Bacilli and actinobacteria also showed changes in band patterns.
Figure 1. DGGE-PCR gel of samples taken for the four treatments. White arrows mark bands unique for the specific treatments. The dendrogram (right) show the relationships according to bands patterns, between populations in each of two samples taken from the four treatments.

**Disease control**

*T. harzianum* T39 or solarization significantly reduced disease severity of *B. cinerea* in detached leaves of cucumber, strawberry and common bean. The combined treatment reduced disease even more, namely by 60%, 95% and 90% for bean, strawberry and cucumber, respectively. Results obtained with whole plants were similar to results from detached leaves (results not shown). 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 (results not shown). After 10 days the combined treatment significantly reduced percentage leaf coverage by about 85%, compared to untreated. Some of the results were reported earlier (Okon Levy et al., 2004).

Figure 2. Effect of isolates of *Bacillus* sp. on bean grey mould. *Bacillus* sp. were isolated from soil treated by solarization, grown on nutrient agar and applied to the roots of bean plants. Disease severity was evaluated on leaves of the treated plants. Isolates 1, 4, 7, 10 and 12 were significantly different from the untreated control ($P \leq 0.05$).
It was suspected that the microbial populations developed in the soil in response to soil solarization and *Trichoderma* treatments may have biocontrol and induced resistance capabilities. Indeed, such bacterial isolates were capable of inducing resistance when introduced to a fresh plant growth medium in which plants were grown (Figure 2). A significant disease control of *B. cinerea*, by applying to the root zone, for Bacilli collected from solarized soil, was observed.

It can be concluded that treatment with solarization or *T. harzianum* resulted in microbial changes in the plant rhizosphere and in induced resistance to *B. cinerea* and *S. fuliginea*. 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, 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 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**


Collimonas fungivorans and bacterial mycophagy

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Introduction

Bacterial mycophagy is defined as the ability of bacteria to grow at the expense of living fungal hyphae. A few years ago de Boer et al. (2001) demonstrated that a group of chitinolytic bacteria isolated from slightly acidic dune soils on the island of Terschelling, The Netherlands, could grow at the expense of the mycelium of selected dune soil fungi. These so-called Ter isolates (22 in total) have been shown to belong to the β-subclass of Proteobacteria, based on genetic and biochemical properties. Further taxonomic characterization showed that they constituted a new genus, Collimonas (de Boer et al., 2004), within the family Oxalobacteraceae (fig. 1) and some of the isolates were assigned to the new species C. fungivorans.

Figure 1. Phylogenetic tree showing the relationship between 12 unique 16S rDNA sequences of Collimonas spp. and other members of the family “Oxalobacteraceae”. The 16S rDNA sequence of Ralstonia solanacearum ATCC 11696T was used as outgroup. The unrooted tree was constructed by the neighbour-joining method. Asterisks indicate nodes with bootstrap values of 950 or higher (1000 replicates). Bar, 0.01 nucleotide substitutions. Modified from de Boer et al., 2004.
Results and discussion

In experiments applying nutrient-limiting, soil-like conditions the growth of *Collimonas* bacteria was quantified in the presence and absence of fungi. Seventeen different *Collimonas* strains were used individually to inoculate microcosms containing purified sand (fig. 2). Agar plugs with the fungi *Mucor hiemalis* (zygomycete), *Fusarium culmorum* (hyphomycete) and *Chaetomium globosum* (ascomycete) were added to these microcosms and after 2.5 weeks, the numbers of *Collimonas* (expressed as colony forming units, cfu) were determined in sand samples collected in the mycelial zones. To test whether all strains were able to grow in the sand, they were inoculated into sand amended with N-Acetyl-D-glucosamine (1 mM in 5% moisture content of the sand). For all tested strains growth on N-Acetyl-D-glucosamine and in the mycelial zones of the three fungi was observed. The increase of the cell numbers depended on the introduced fungi. For all tested *Collimonas* strains the best stimulation was observed with *M. hiemalis*. Additionally the growth response of the different isolates to one fungus varied clearly (fig. 2).

![Figure 2](image_url)

**Figure 2.** Growth of 17 *Collimonas* spp. in purified sand in the mycelial zones of *Mucor hiemalis*, *Fusarium culmorum*, *Chaetomium globosum* and in sand amended with N-Acetyl-D-glucosamine (AG). The experiments were performed in petri dishes containing 50 g sand as described in de Boer et al. (2001) but each experiment was carried out in two replicates. Samples were taken 2.5 weeks after the introduction of the fungal inoculum. Each bar represents the average of 17 single experiments using 17 different *Collimonas* strains, the values are corrected by subtracting the number of grown bacteria in control sand without addition of fungi and AG, respectively. The standard deviations are calculated from the data obtained by all strains and indicate the different response of single strains to the fungi.

For a mixture of 9 *Collimonas* strains the dynamics of the bacteria in purified sand in the mycelial zones of *C. globosum* and *M. hiemalis* was determined in time (de Boer et al., 2001). Additionally the extension of the mycelia were observed. About two weeks after the mycelia had reached their maximum extension, the strongest stimulation of the bacteria was observed (fig. 3A; de Boer et al., 2001).

The chitinase inhibitor allosamidin was used to investigate whether chitinases play a role in the attack of the fungal hyphae by *Collimonas* spp. in sand microcosms (fig. 3B, de Boer et al., 2001). Although a significant reduction of growth of *Collimonas* spp. in mycelial
zones of *F. culmorum* and *C. globosum* was observed when allosamidin was added to the sand, the inhibition was only partial. This supports the hypothesis that besides chitinases other antifungal factors (other lytic enzymes, antibiotic substances) are involved in the attack of the fungi by *Collimonas* species. No effect of allosamidin was observed for growth on *Mucor* hyphae. This may be due to the different composition of the cell wall of this fungus containing chitosan rather than chitin.

![Figure 3](image)

**Figure 3.** A: Increase of the number of *Collimonas* (in colony forming units [cfu]) in the mycelial zones of *C. globosum* and *M. hiemalis*. Arrows indicate the times when the mycelial extension stopped. Standard deviations are indicated by error bars (n=4) (de Boer et al., 2001). B: Effect of the chitinase inhibitor allosamidin (10µM in soil solution) on the growth of *Collimonas* spp. in purified sand in mycelial zones of *F. culmorum*, *C. globosum*, and *M. hiemalis* after four weeks after introduction of fungal inoculum (n=4). Statistical significant differences (P<0.05) between allosamidin and control treatments are indicated by asterisks. Modified from de Boer et al., 2001.

Investigations to elucidate the interesting mechanism of bacterial mycophagy using *Collimonas fungivorans* as model organism are underway. For this we use a genomic approach that involves sequencing the genome of *Collimonas fungivorans*, analysis of large-insert genomic libraries (Leveau et al., 2004), and screening for loss- or gain-of-function phenotypes in transposon mutant library clones.

**References**

Endophytes as source of efficient biological agents

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Abstract: Endophytes are plant-associated bacteria that live inside plants and show neutral or beneficial interaction with the host plants. The biocontrol potential of endophytic bacteria isolated from field-grown potato plants was evaluated in a hierarchical combination of assays using the soil-borne plant pathogen *Rhizoctonia solani* Kühn as target. The pathogen is one of the most important soil-borne pathogens and responsible for yield losses of economically important crops worldwide. Control of *R. solani* is difficult, because of its saprophytic and parasitic properties. Thus, the objective of the study was to select effective biological control agents (BCAs) useful for different crops. The strains were characterized by their antagonistic mechanisms *in vitro* against different anastomosis groups of *R. solani* as well as their effectiveness in disease suppression *in vivo*. In summary, 18 bacteria were selected based on antagonistic activity in dual culture and enzyme activity (chitinase, β-1,3-glucanase, protease) *in vitro*. The disease depression effect of these strains was further tested against *R. solani* on potato, lettuce and sugar beet under controlled cultivation conditions, favourable for the disease development of *R. solani*. Ten BCAs reduced the disease development on potato, seven on lettuce and ten on sugar beet. But only three isolates showed effects against *R. solani* on all three crops. These three bacterial BCAs were tested in field experiments on potato and lettuce. The plant growth of both was significantly diminished by *R. solani* in the field. The application of the bacterial BCAs reduced significantly the disease severity on lettuce and potato. The reduction of disease severity correlated with an increasing yield in the treatments due to the bacterial BCAs on both crops.

Key words: endophytes, biological control, *Rhizoctonia solani*, lettuce, potato

Introduction

The widespread soil-borne pathogen *Rhizoctonia solani* Kühn causes yield losses on an extremely wide host range of important horticultural and agricultural crops worldwide (Adams 1988). Control of Rhizoctonia diseases is difficult because of the ability to survive as sclerotia under adverse soil environmental conditions for many years, its ability of saprophytic activity, and its extremely wide host range. Fungicides were mainly used as control methods. However, the European Union decided that 60% of the chemical pesticides that were allowed in 1996 were banned in 2003. In addition, for some crops, especially for horticultural ones, no fungicide exists. That is why new alternative strategies to control the pathogen are urgently necessary.

An environmentally friendly and sustainable alternative to protect plants against soil-borne pathogens is biological control using antagonistic microorganisms as biocontrol agents (BCAs) (Whipps 1997; Emmert & Handelsman 1999). In several studies fungal antagonist against Rhizoctonia diseases were investigated (Lewis & Larkin 1998; Ross et al. 1998; Van den Boogert & Luttikholt 2004). However, only few bacteria have been reported to control Rhizoctonia, e.g. *Bacillus licheniformis* (Ahmed et al. 2003), *Burkholderia cepacia* (Cartwright et al. 1996), and *Lysobacter enzymogenes* (Kilic-Ekici & Yuen 2003). Other
studies demonstrated the use of endophytically living bacteria against \textit{R. solani} as a potentially powerful control strategy under greenhouse conditions (Pleban et al. 1995; Cho et al. 2003). 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 harm the plant. However, only a few commercial biocontrol products based on antagonistic rhizobacteria and none based on endophytes are currently available on the market (Whipp 1997).

The objective of our study was the selection and evaluation of a biological control agent (BCA) against \textit{Rhizoctonia} diseases. The bacteria strains were selected based on a hierarchical combination of \textit{in vitro} and \textit{in vivo} assays. The effectiveness of the best strains was evaluated against Rhizoctonia diseases black scurf in potato and bottom rot in lettuce in field experiments.

**Materials and methods**

**Screening of potential \textit{Rhizoctonia} antagonist**

A number of 434 bacterial strains isolated from microenvironments like rhizosphere and endorhiza of potato during previous studies (Berg et al. 2001; Krechel et al. 2002) were evaluated against \textit{R. solani}. The traditional dual culture assay was used to find out the antagonistic potential against \textit{R. solani}. The ability to produce cell wall-degrading enzymes (chitinase, \(\beta\)-glucanase and protease) was investigated \textit{in vitro} from isolates with an antagonistic activity. Parallel to this, the ability to promote plant growth was analysed in a newly developed assay in microtiter plates using lettuce seedlings in phytochamber, and by the production of indole-3-acetic acid. Efficient isolates were identified by sequencing of 16S rDNA to avoid a further work with potentially pathogenic species.

**Growth chamber experiments**

**Potato.** The disease suppression effect of BCAs was determined on potato sprouts growing from seed tubers (‘Exquisa’) in a sand-substrate (‘Fruhsdorfer Einheitserde’ type P) mixture (SSM, 1:1 v/v) in 12 x12x20 cm plastic pots. The whole SSM in each pot was treated with 180 ml of bacterial suspension (10^8 CFUml\(^{-1}\)) of each BCA. The pathogen inoculum was placed 2 cm above a seed tuber and covered with the SSM. Each treatment included six replications in a randomized design. Disease severity (DS) was assessed on a scale from 1 to 5 (1 = no symptoms, 2 = lesions<5 mm, 3 = lesions>5 mm, 4 = strong damages on whole sprout, and 5 = all sprouts dead) after 3 weeks incubation at 20 °C in the dark.

**Lettuce.** The control effect of the BCAs against \textit{R. solani} on lettuce (‘Daguan’) was tested in growth chamber (16-h light and 8-h dark photoperiod, light input 500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) with 70% and 90% relative humidity respectively). Lettuce seedlings were treated with each 5 ml of bacterial solution (10^7 CFU ml\(^{-1}\)) 1 week before planting in pots and 1 day after planting by drenching with 20 ml of bacterial suspension (10^7 CFU ml\(^{-1}\)). The pathogen was inoculated at 2-3 leaf stage at planting time. Each treatment included 6 replications with 4 plants each arranged in a randomized design. Three weeks after pathogen inoculation, the dry mass (DM) of plants were measured.

**Field experiments**

**Potato.** The bacterial BCAs B1, B2 and B4 were tested against black scurf disease on potato ‘Exquisa’ in the field (soil type, loamy sand). Each treatment included 6 replications with 21 potato tubers planted 30 cm apart within a row and each row is 65 cm apart in a randomized design. The plots were fertilized with 150 kg N ha\(^{-1}\) 2 weeks before planting. The seed tubers were bacterized (126 tubers with 250 ml bacterial suspension,10^8 CFU ml\(^{-1}\)) before planting.
A second treatment with BCA was performed 2 weeks after planting (100 ml suspension/plant, $10^8$ CFU ml$^{-1}$). The emergence of seed tubers and the marketable potato yield at harvest were assessed and the disease severity (DS) on tubers with *Rhizoctonia* sclerotia on a scale from 1 to 4 ($1 =$ without sclerotia, $2 = <1\%$ sclerotia infestation, $3 = 1\%-5\%$ sclerotia infestation, and $4 = >5\%$ sclerotia infestation). The average DS was calculated from 120 (20 tubers per replication) randomly selected potato tubers.

**Lettuce.** The effect against bottom rot on lettuce was tested in a field (100 m x 24 m, sandy loam, 11.1 plants per m$^2$), naturally infested with *R. solani* (Grosch et al. 2004). The treatments were arranged in a randomized design with 7 replications. Fertilizer was used 1 d before planting (100 kg N ha$^{-1}$ and 120 kg K ha$^{-1}$). Overhead sprinkler irrigation was applied immediately after planting and then as required to supplement rainfall and maintain good crop growth.

Lettuce seedlings ‘Nadine’ were transplanted by hand in the field. The lettuce (150 seedlings) were drenched with a bacterial suspension (6 ml/plant, $10^7$ CFU ml$^{-1}$) first 7 d before planting at the 2-3 leaf stage. The second application was carried out 5 d after planting with 1.0 l of bacterial suspension ($10^9$ CFU ml$^{-1}$) per m$^2$ soil surface. The lettuce plants were harvested after a cultivation time of 6 weeks. At harvest the dry mass was measured and the DS assessed on a scale from 1 to 7 according to Kofoet et al. (2001).

![Figure 1](image1.png)

Figure 1. Effect of bacterial BCAs (*P. fluorescens* B1, *P. fluorescens* B2 and *S. plymuthica* B4) on dry mass (DM) of lettuce ‘Nadine’ and disease severity (DS) of bottom rot on average of two field experiments. The mean in a columns of DM followed by the same letter is not significantly different according to Tukey’s test ($P=0.05$) and in columns of DS according to Kruskal Wallis test ($P<0.05$). DS was assessed on a scale from 1 to 7 ($1 =$ healthy plants, $3 =$ low, $5 =$ moderate and $7 =$ severe symptoms of bottom rot).

**Results and discussion**

Using a hierarchical combination of assays *in vitro* it was possible to select 20 potential BCA against *R. solani*. The selected BCA showed antagonistic activity against various *R. solani*
isolates in vitro. All these isolates produce protease. However, only a low percentage of glucanolytic and chitinolytic isolates was found (Faltin et al. 2004). The production of inhibition zones in dual culture is due to the production of antibiotics, toxic metabolites, or siderophores (Swadling & Jeffries 1996).

Based on the results of the climate chamber experiments on potato and on lettuce, three efficient bacterial antagonists (Pseudomonas fluorescens B1, P. fluorescens B2 and Serratia plymuthica B4) against Rhizoctonia were selected, which were able to suppress the disease development caused by R. solani as well as on potato and lettuce. Whereas the strain B1 was originally obtained from the potato rhizosphere, the strains B2 and B4 were endophytically living bacteria strains, isolated from the endorhiza of potato. Other in vitro selected strains reduced the DS on lettuce but not on potato and reverse in the climate chamber experiments. The goal of the screening was to select BCA efficient against Rhizoctonia diseases on different crops. So the best three antagonists with disease suppression effect on both crops were further tested under field conditions on lettuce and potato.

All three candidates were able to decrease the disease severity (DS) on lettuce in two field experiments. The reduction of DS correlated with increasing dry mass in the treatments with the bacterial BCAs on lettuce (Fig. 1). The results of climate chamber experiments showed, that R. solani has a negative effect on the lettuce growth. The BCAs were able to limit significantly the influence of the R. solani infection on lettuce growth in the field.

The disease suppression effect of the bacterial BCAs was also tested on potato in field plots. A sclerotia infestation of approximately 1% was assessed on average in the control treatment (Figure 2). However, in all BCA treatments, the number of healthy tubers was significantly higher compared with the control. So, the disease severity (DS) on harvested potato tubers with R. solani was significantly decreased in all treatments with the BCAs. The reduction of the DS correlated with a higher marketable potato yield.

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. Therefore, the endophytic candidates are promising biological control agents. The effect of the bacterial BCAs has to be
checked in further field experiments. Through an optimal formulation and application density and frequency it might be possible to improve the effectiveness of the BCAs.

References


Towards indicators of soil biological quality: use of microbial characteristics

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Abstract: The aims of this study were to evaluate the impact of 2 cultural practices on diverse soil characteristics, and to establish relationships between these descriptors, in order to identify indicators of the soil biological quality. The experimental plot received either a cattle manure compost amendment, or was treated by biofumigation. Physicochemical and biological data, microbes and nematodes community structure and the soil receptivity to *Rhizoctonia solani* damping-off of carrot were assessed during a whole cultural year. Correspondences were analysed by Principal Component Analysis. Significant changes were observed. Biofumigation induced the strongest effect. The organic amendment resulted in a weaker effect. We are still working on the establishment of relationships for the identification of indicators of the soil biological quality.

Key words: biofumigation, organic amendment, T-RFLP, PCA, field survey

Introduction

Soil quality has been defined, more than 10 years ago, by Doran and Parkin (1994). Until recently, mainly the chemical and physical components have been used to describe soil quality, since the biological part is much more difficult to evaluate. However, its role should not be underestimated. To assess the biological quality of a soil, there is a need to identify indicators. It is not realistic to imagine that one unique indicator would be representative of the global, complex, soil biological quality. It is why we are looking for a bunch of indicators. The microbial communities are involved in the soil functioning. They are also highly sensitive to perturbations, for example the cultural practices. It is why their characteristics are potential indicators of the soil biological quality.

The aims of our work were to evaluate the impact of 2 cultural practices (organic amendment and biofumigation) on soil characteristics, microbes and nematodes communities and *Rhizoctonia solani* soil receptivity level. We then want to establish relationships between these descriptors to finally identify which descriptors would be the best indicators of soil biological quality.

Material and methods

Experimental plot and sampling

The experimental plot is located in Bergerac (24, France). It was divided in 3 sub-plots. The control plot (Té) was conducted in conventional agriculture. The second sub-plot (MO) received 30 tons per hectare of composted cattle manure. Biofumigation with fodder radish and plastic film tarping was applied on the third sub-plot (Bd). Twenty-one soil samples were...
collected in each sub-plot at 3 key points during the cropping sequence (before treatment, before and after carrot culture). The results presented correspond to those of the second sampling date (T1), one week before carrot sowing.

**Analyses**

Physicochemical characteristics of each soil sample were measured. Densities of cultivable bacteria and fungi were evaluated by the soil suspension-dilution technique. Microbial biomass was assessed by the chloroform fumigation-extraction method. Basal respiration of fresh soil was measured by sampling in closed bottles and gas chromatography. Direct nematodes counting and identification were realised after soil extraction. The microbial community structures were assessed by Terminal Restriction Fragment Length Polymorphism on the 16S (bacteria) or 18S (fungi) ribosomal DNA sub-unit. Inoculation of plantlets with the different soil samples and *Rhizoctonia solani* sand inoculum enabled us to measure the soil receptivity to *R. solani* damping-off of carrot. Crop yield and quality were rated at time of harvest.

Principal component analysis was chosen to analyse the data. Permutation tests were performed to detect significant differences between the 3 treatments and between-class PCA were applied on the data, with the means of each plot.

**Results and discussion**

**Physicochemical analyses**

Amended and biofumigated soils are well separated from control soils on PC1. There were more magnesium, sodium, nitrogen, carbon and organic matter in these soils. Organic amendment and biofumigation treatments are separated from each other on PC2.

![Figure 1. Between-plot PCA of the soil physicochemical characteristics at T1. (A) Factorial plan PC1xPC2. (B) Correlation circle PC1xPC2 of the variables.](image)

**Biological analyses**

This PCA was performed with the fungal and bacterial densities, microbial biomass, soil respiration rate and the results of the *R. solani* receptivity bioassay. A strong effect of the biofumigation is highlighted. The permutation test revealed a significant effect of the treatment on the structure of these data. The biofumigated soil had significantly higher microbial densities and activity. This was correlated with a lower death rate of the carrot
plantlets inoculated with *R. solani* in this soil. The biofumigated soil was more suppressive to
*R. solani* damping-off of carrot.

**Nematode communities**

The cultural practices didn't change the qualitative composition of the nematode communities. The same 12 genera were found in the samples along the year. However, the organic amendment and the biofumigation had a significant effect on the nematode densities and structure of the community. On PC1, biofumigation was well separated from control. This was due to more important densities of *Trichodorus*, *Xiphinema* and *Aphelenchus*. The organic amendment was separated from the 2 other sub-plots on PC2, due to the *Ditylenchus* densities.
**Bacterial and fungal community structures**
The effect of the cultural practices was significant on the bacterial community structure. Biofumigated soil was separated on PC1 and amended soils on PC2.

For fungal community structure, the differences between the 3 sub-plots were less pronounced and only the biofumigated plot was well separated from the 2 others.

![Figure 4. Between-plot PCA of the bacterial (A) and fungal (B) community structure data. Factorial plans PC1xPC2 of the plot centers of gravity.](image)

**Field observations**
The quality and disease rating of the carrot harvest showed no significant difference between the 3 sub-plots (data not shown). However, the mean weight of the carrots was higher in the compost amended soil. But in this plot there were more forked carrots and more *Pythium* spots on the carrots.

**Conclusions**
The plot was not fully homogenous at the beginning of the experiment (data not shown). However, strong changes were detected after the application of the cultural practices. Biofumigation had the most important impact. The increase in microbial densities and activity and the correlated decrease in the soil receptivity to *R. solani* damping-off can not be explained by a unique mechanism such as a biocidal effect of isothiocyanates. It is rather a combination of mechanisms due to the amendment with fresh organic matter, the moderated solarization induced by the tarping, and the disinfectant effect of isothiocyanates. Concerning compost amendment, modifications were also observed, but to a lesser extent. Repeated applications might be needed to obtain strong changes of the soil biological characteristics. The same cultural practices have been applied for the second year, and the same parameters are being measured. We expect to observe the same or even stronger effects. With 2 years of data, we will determine the most useful parameters that could be indicators of the biological quality of soil.

**References**
Interactions between arbuscular mycorrhizal fungal hyphae and soil bacteria – Effects of hyphal exudates on bacterial diversity

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Abstract: In addition to increasing the nutrient absorptive surface area of their host plant root systems, the extraradical hyphae of arbuscular mycorrhizal (AM) fungi also provide a direct pathway for translocation of photosynthetically derived carbon to microenvironments in the soil. This provision of energy-rich compounds, coupled with the relatively large surface area of the hyphae, suggests that mycorrhizal mycelia may be an important niche for bacterial colonisation and growth. What effect mycelial exudates may have on microbial diversity and the activity of the microorganisms is still unknown. Mycelial exudates of Glomus sp. MUCL 43205 were collected from split-plate in vitro systems of transformed, mycorrhizal clover roots. A bacterial community extracted from soil was incubated in the presence or absence of exudates to determine their effects on bacterial community structure. Initial and post-exposure bacterial communities were characterised by T-RFLP analysis and 16S DNA sequencing. We also determined bacterial viability using Live/Dead® BacLight™ stain. Both the bacterial diversity and the relative abundance of some individual taxa were higher in the mycorrhizal treatment compared to the non-mycorrhizal treatment. We also found that the proportion of vital bacteria increased in the presence of AM fungal exudates. Initial results suggest that some taxa were exclusively associated with exposure to AM fungal exudates. The results support the idea that AM fungal exudates may influence both soil bacterial diversity and abundance.

Key words: arbuscular mycorrhiza, bacterial community, extraradical mycelium, hyphal exudates, T-RFLP

Introduction

Arbuscular mycorrhizal (AM) associations are formed between the roots of most terrestrial plants and fungi of the phylum Glomeromycota (Schussler et al., 2001). The extraradical mycelia (ERM) of these fungi explore the soil for resources, thereby increasing the nutrient absorptive surface area of their host plant root systems. In addition it has been estimated that up to twenty percent of photoassimilated carbon is invested by the plant in the mycorrhizal symbiosis (Johnson et al., 2002). Some of this carbon is exuded through the ERM in the soil. These exudates, along with the hyphae themselves, can be used by microorganisms as a source of energy and nutrients. The large surface area provided by the ERM in the soil and its supply of energy-rich carbohydrates therefore represents a potentially important niche for bacterial colonisation and growth (Johansson et al., 2004).

Different soil bacteria may differ in their ability to colonise the surface of AM fungal hyphae, some being efficient hyphal colonisers. In a previous study we found that several soil bacterial strains showed a preference for colonising either living or dead AM fungal hyphae (Johansson et al., submitted manuscript). Attachment and colonisation of certain bacteria to living AM fungal hyphae may be an important prerequisite for interactions between these microorganisms, with implications for nutrient supply and biocontrol in sustainable agriculture (Johansson et al., 2004).
The aim of the current study was to determine whether AM fungal mycelia influence bacterial diversity and community composition. These parameters are presumably linked to ecosystem function and influence microbial processes that occur in the soil. It is possible that some bacteria and AM fungi have co-evolved, and that there are specialised bacterial communities associated with AM fungi. Changes in abundance of certain bacterial taxa in response to hyphal exudates should provide new information on how they interact with the AM fungi.

Material and methods
We used two-compartment Petri dish systems with non-mycorrhizal and mycorrhizal (Glomus sp. MUCL 43205) ribosomal T-DNA-transformed roots of white clover (Trifolium repens L.). Liquid carbon free minimal media, which had been exposed to the AM fungal extraradical mycelium, but not to the roots, for one month, was harvested and incubated with a bacterial community, freshly extracted from field soil. During incubation, bacteria were sampled at time intervals, and changes in soil bacterial community structure over time was analysed using Terminal-Restriction Fragment Length Polymorphism (T-RFLP) of bacterial 16S rDNA. In addition, the chemical composition of AM-fungal exudates present in the liquid media prior to addition of bacteria was analysed using Proton Nuclear Magnetic Resonance (H-NMR) analysis.

Results and discussion
Exudate composition
The H-NMR analysis revealed differences between the chemical composition of the liquid medium from the mycorrhizal treatment and that from the non-mycorrhizal treatment (Fig. 1). The exudate from the mycorrhizal treatment contained different forms of glucose, as well as formic and acetic acid.

Changes in bacterial community composition
There was an overall increase in bacterial numbers during the experiment. And there was a significantly higher proportion of live bacteria in the mycorrhizal treatment after 48 hours. T-RFLP analysis revealed that the bacterial community structure changed over time. When considering only the most common bacterial taxa, each representing at least one percent or more of the total community, we could see that the bacterial groups that occurred at the time of
incubation had been replaced by different groups of bacteria at 20 and 48 hours. At 48 hours there was a higher diversity of bacterial taxa, both in terms of a larger number of bacterial groups and as a higher community evenness in the mycorrhizal treatment (Fig. 2).

![Image of bacterial community profile](image)

The observed changes in bacterial community structure can be explained by several factors. Carbohydrates released by the fungus can be used by bacteria as a source of energy. However, bacteria may also compete with each other for these resources. Bacteria better adapted to the environment around the fungal extraradical mycelium increase in abundance. However, as carbohydrates released by the fungal mycelium may serve as energy for bacteria, exudation may also alleviate competition, allowing more bacterial groups to co-exist, and this could explain the higher number of taxa and increased community evenness that was observed in the mycorrhizal treatment. There could also be a more selective influence of hyphal exudates on certain bacterial groups. Organic acids could play a role in this process, however it is also possible that other compounds, for example antibiotics, are involved.

Two genera of mycobacteria (*Actinomyces* sp. and *Mycobacterium* sp.) and *Arthrobacter* sp. were among the most common bacteria represented in the mycorrhizal treatment in the end of the incubation period. Both of these groups comprise species that are ubiquitous in soils. Mycobacteria, many of which are pathogenic to animals and humans, typically are very acid tolerant. Some *Arthrobacter* spp. are tolerant to organic soil pollutants, and have therefore been used as bioremediation agents for restoring contaminated soils (Westerberg et al, 2000). Evidently, the mycorrhizal mycelium is capable of producing organic acids, which may have either positive or negative effects on different organisms in the mycorrhizosphere. It is possible that above described bacteria are represented in the mycorrhizal treatment simply because they are able to detoxify the environment surrounding the mycelium.

Although a less abundant bacterial group in this study, *Burkholderia* sp. responded positively to AM mycelial exudates. Representatives from this genus have previously been described as obligate endosymbionts in some AM fungi (Bianciotto et al., 2004), however their function is currently unknown. Other recent studies have shown that *Burkholderia* spp.
efficiently colonise the exterior of fungal structures, and in some cases enhance AM fungal spore germination (Levy et al., 2003), possibly by immobilising or detoxifying compounds that would otherwise inhibit germination.

**Conclusions**

The results of this preliminary study suggest that both bacterial diversity and the relative abundance of some individual taxa were higher in the mycorrhizal treatment compared to the non-mycorrhizal treatment. We also found that the proportion of vital bacteria increased in the presence of AM fungal exudates. Our initial results suggest that some taxa were exclusively associated with exposure to AM fungal exudates and support the idea that AM fungal exudates may influence both soil bacterial diversity and abundance.

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Physiological and physico-chemical factors modulating ISR elicitor production by *Pseudomonas putida*

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**Abstract**: A better understanding of biotic and environmental factors that regulate the production of active metabolites by beneficial rhizobacteria is crucial for optimising biocontrol under practical conditions. In this study, we wanted to evaluate the effect of some parameters on the production of the plant defence elicitor synthesized by *Pseudomonas putida* BTP1. This molecule is clearly dependant of the secondary metabolism and chemostat experiments showed that the elicitor is more efficiently produced at a very low cell growth rate. On another hand, the presence of free amino acids in the medium is necessary to obtain an optimal NABD production. A specific positive effect of phenylalanine was evidenced in pulsed continuous cultures suggesting that this residue could play a role as a precursor and/or act as a regulator in the biosynthetic pathway.

**Key words**: rhizobacteria, induced systemic resistance, biological control

**Introduction**

The application of some non pathogenic bacteria living in association with roots is of interest for the biological control of plant diseases. These beneficial rhizobacteria can inhibit pathogen growth via diverse mechanisms such as antibiotic production, site exclusion or siderophore-mediated iron competition uptake (Whipps 2001; Zahir et al. 2004). However, selected strains can also stimulate defense mechanisms into the plant tissues, rendering the host less susceptible to a subsequent pathogen attack. As it is long-lasting and effective in distal tissues, this phenomenon was termed induced systemic resistance (ISR, (Bakker et al. 2003; Van Loon et al. 1998) by analogy with the pathogen-derived SAR (Durrant and Dong 2004). In comparison with SAR, little is known about the molecular events associated with ISR regarding both defense mechanisms in plant tissues and microbial elicitors of the phenomenon. Elicitor structures identified so far can be classified as cell surface components like flagella or lipopolysaccharides (Meziane et al. 2005; Van Loon et al. 1998), antibiotics (pyocyanin/pyochelin, 2,4-diacetylphloroglucinol) (Audenaert et al. 2002; Lavicoli et al. 2003) and iron-regulated metabolites such as salicylic acid, pyochelin or pyoverdines (De Meyer et al. 1999; Maurhofer et al. 1998; Meziane et al. 2005).

In this context; we have studied for several years a *Pseudomonas putida* strain (BTP1) for its ability to trigger ISR in cucumber and bean. Our investigations led to the conclusion that metabolites previously identified as *Pseudomonas* determinants for ISR, were not involved in systemic resistance triggered by BTP1 (Ongena et al. 2002) and we have characterized this new ISR determinant as a N-alkylated benzylamine derivative (NABD) (Ongena et al. 2005).

The overall physiological status and activity of bacterial inoculants used as biopesticides is of prime interest for the efficacy of their introduction in the environment. In
the rhizosphere, microbial biomass, activity and community structure are highly influenced by specific physicochemical and biological characteristics prevailing in this habitat. In this study, we wanted to evaluate the effect of some physiological and physico-chemical factors influencing the synthesis of this elicitor by the strain.

**Material and methods**

**Culture conditions**

Cells of *Pseudomonas putida* BTP1 were grown either in 100 ml agitated erlenmeyer flask filled with 50 ml of culture medium (120 rpm, 30°C) or in 2 L- glass reactors (B-Braun) filled with 1.5 L of casamino acids medium (CAA, bacto-agar, 12 g l⁻¹, casamino acids, 5 g l⁻¹; MgSO₄.7H₂O, 0.25 g l⁻¹; K₂HPO₄, 0.9 g l⁻¹). The temperature was maintained at 30°C and the pH value was regulated at 7.0 ± 0.2 by addition of H₃PO₄ (3N). Aeration rate and agitation were fixed respectively at 0.1 vvm and 300 rpm for batch or 600 rpm for chemostat cultures. These cultures were inoculated with a 2% volume of 16 h-subcultures realized in the same culture medium. In order to minimize contaminating iron traces, glassware and bioreactors were rinsed with nitric acid (0.5 N) and water used to prepare all media was purified by the Milli-Ro System (Millipore, Massachusetts). For chemostat culture, fresh medium was fed into the reactor using a peristaltic pump to control the dilution rate. The volume of the culture was maintained constant by the use of an overflow device. Oxygen tension was measured with a polarographic oxygen electrode (Mettler toledo, Switzerland). Bacterial development was measured by plating on agar-solidified Cell concentration was evaluated on the basis of optical density at 540nm knowing that a OD₅₄₀ value of 1 corresponds approximately to 4.5 x 10⁸ cells/mL growing exponentially. NABD was extracted on C18 solid phase cartridges (Isolute, Alltech) from culture supernatants after cell removal by centrifugation and its concentration was determined by reverse phase HPLC as described in (Ongena et al. 2005).

**Influence of carbon source, minerals and amino acids**

Beside CAA, several media were used to test the effect of carbon source on elicitor production. The composition of King’s B medium is tryptone 10g/L, Proteose peptone 10g/L, K₂HPO₄ 0.9g/L, MgSO₄.7H₂O 0.25g/L and glycerol 10g/L. The other substrates (mannitol, glucose, fructose, mannose, glycerol, succinate, glutamate) were tested by adding a concentrated solution to a minimal medium composed by MgSO₄.7H₂O 0.2g/L, K₂HPO₄ 0.9g/L, (NH₄)₂SO₄ 1g/L to obtain a final concentration of 10g/L. To test the influence of oligoelements, the following minerals CoCl₂ 6(H₂O), CuSO₄ 5(H₂O), ZnSO₄ 7(H₂O), FeSO₄ 7(H₂O), MgSO₄ 7(H₂O), NaCl and (NH₄)₆Mo₇O₂₄.7H₂O were added to the CAA medium to have a final concentration of 0.1mM. The effect of individual amino acids was tested in 100 mL culture flasks by adding 100μL of a concentrated solution of phenylalanine, tyrosine, lysine, isoleucine or tryptophane to obtain a final concentration of 0.1g/L in the medium. For testing the effect of phenylalanine pulse in chemostat, 5mL of a concentrated solution of the amino acid was steriley injected into the bioreactor to have a final concentration of 1g/L in medium.

**Results and discussion**

**Influence of growth rate**

The NABD elicitor was isolated from supernatant obtained after growth of *Pseudomonas putida* BTP1 in the iron-poor Casamino acids medium (CAA) commonly used to cultivate such organism. Growth and NABD production in this medium were thus first monitored in time during batch cultures realized in flask or in bioreactor with regulated pH and air flow.
NABD is not detectable during the first 16 h of culture corresponding to the exponential growth phase (Figure 1). Its production only starts once cells entered the stationary phase. Elicitor production is not markedly influenced by pH, regulated at 6.5 in bioreactor but which reached a value of 8.5 at the end of the flask culture, or by dissolved oxygen concentration which is higher in the aerated bioreactor.

![Figure 1. Cell concentration and NABD production in flask and bioreactor. Cell concentration was determined by optical density at 540 nm and NABD production was estimated by HPLC.](image)

This molecule is obviously not necessary for bacterial growth and could thus be dependant of the secondary metabolism. In order to test this hypothesis and to further investigate the influence of the growth rate on NABD production by *P. putida*, cells were cultivated in the chemostat culture mode at different dilution rates. Generally, the production of microbial cell mass is limited by the restricted availability of a particular nutrient. In batch culture, the exhaustion of a specific nutrient terminates the exponential growth phase, whereas in chemostat culture the biomass concentration is usually controlled by the permanent limitation of a single defined nutrient, soluble iron in our case.

![Figure 2. Elicitor production in nanogram per cell unit in flask (flask), bioreactor (bioreactor) and chemostat (chem.). dilution rate used were 0.1 (chem. D 0.1); 0.3 (chem. D 0.3) and 0.6 (chem. D 0.6). Elicitor production (in ng/10^8 Cells) was estimated by HPLC chromatography. Values with different letters are significantly different at P = 0.05 according to Fisher's least significant difference test.](image)
As shown in Figure 2, our results revealed that the higher growth rate is, the lower NABD production is, strongly suggesting that a low cellular growth rate is necessary for an efficient synthesis of the molecule. Root-colonizing pseudomonads have maximal doubling times about 10-times slower than that in rich laboratory media. It implies that bacterial growth in the rhizosphere is limited by some available nutrients among which nitrogen source and oxygen may be the most important. In general, microorganisms growing optimally in balanced, nutrient-rich media generally use metabolic energy to synthesize cellular components and to grow, not for secondary metabolism. When growth is restricted, either because of nutrient limitation or at high cell density, certain microorganisms switch on secondary metabolism (Haas and Defago 2005). In the case of strain BTP1, the evolution of bacterial population on roots has not yet been thoroughly studied but, as observed for other strains, a residence phase can be rapidly obtained where the established populations reaches the maximum size relative to the root weight and its further growth couples with slow plant growth. It may thus be assumed that most of the BTP1 cells are multiplying in the rhizosphere at a very low growth rate around or below 0.1 h\(^{-1}\) compared to \(\mu_{\text{max}} = 0.7\) h\(^{-1}\) observed in the synthetic CAA medium. The corresponding physiological state of cells on roots should thus be conducive for an efficient synthesis of the NABD elicitor.

**Influence of carbon source and minerals**

In order to test the effect of carbon source on elicitor synthesis, cells were grown in the presence of various sugars and organic acids that are commonly found in root exudates. The levels of NABD production measured at the end of the cultures are compared with the one measured in CAA in Table 1. It clearly appeared that the presence of free amino acids in the medium is necessary to obtain an optimal NABD production. The rhizosphere is an habitat in which living roots release a range of low-molecular-weight substrates, including sugars, organic acids and amino acids. These last compounds may represent an important part of the exudates (Lugtenberg et al. 2002) that could thus be favorable not only for BTP1 cell maintenance and growth but also for the synthesis of NABD. Moreover, the very low production rate observed upon growth on King’s B medium containing protein hydrolysate as sole N source suggests that organic nitrogen is not required for an efficient production of NABD.

Table 1. Influence of various carbon sources on the elicitor production rate by *P. putida* BTP1. Substrates were added to obtain a final concentration of 10 g/L in the medium. Values with different letters are significantly different at \(P=0.05\) (Fisher’s LSD test).

<table>
<thead>
<tr>
<th>Media(^a)</th>
<th>NABD production (\text{(ng/10}^{8}\ \text{CFU)})(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>casamino acids (CAA)</td>
<td>68.2 x</td>
</tr>
<tr>
<td>succinate</td>
<td>12.5 y</td>
</tr>
<tr>
<td>glutamate</td>
<td>3.1 y</td>
</tr>
<tr>
<td>King’s B</td>
<td>4.2 y</td>
</tr>
<tr>
<td>mannose</td>
<td>nd</td>
</tr>
<tr>
<td>Glucose</td>
<td>nd</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.1 y</td>
</tr>
<tr>
<td>Fructose + CAA</td>
<td>35.5 z</td>
</tr>
<tr>
<td>Arabitol</td>
<td>nd</td>
</tr>
<tr>
<td>mannitol</td>
<td>nd</td>
</tr>
<tr>
<td>glycerol</td>
<td>9.8 y</td>
</tr>
<tr>
<td>glycerol + CAA</td>
<td>43.5 x</td>
</tr>
</tbody>
</table>
We also tested the effect of some oligoelements such as Mg, Zn, Cu, Fe, Co, Mo added in the CAA medium and observed that only copper and iron had a significant negative effect on NABD production (data not shown).

**Influence of amino acids**

We further examined the consumption rates of individual amino acids during batch culture of strain BTP1 in the CAA medium. It revealed that some amino acids (Asp, Glu, Pro, Ala, Thr) were preferentially used by the bacterium during the exponential growth phase (data not shown). Other residues such as Phe, Tyr, Trp, Lys or Ile were still present in large amounts in the medium after 24 h and were then slowly consumed during the next 72 h of incubation, corresponding to the NABD production phase (data not shown).

Elicitor production was thus evaluated after growth in CAA medium supplemented with 0.1g/L of these last amino acids in order to test their specific influence on elicitor synthesis. However, a significant increase in NABD production was only observed upon addition of Phe to the medium (Figure 3).

![Figure 3](image1.png)

**Figure 3.** Influence of some amino acids on NABD production by *P. putida* BTP1. The residues were added to obtain a final concentration of 0.1 g/L in the medium. Values with different letters are significantly different at *P* = 0.05 according to Fisher’s least significant difference test.

![Figure 4](image2.png)

**Figure 4.** Effect of individual phenylalanine addition (Pulse) on NABD production by *P. putida* BTP1 in chemostat culture.
This positive effect of phenylalanine on NABD synthesis was then confirmed in Phe-pulsed continuous cultures at a constant cell growth rate of 0.15 h⁻¹. Addition of Phe resulted in a transient 9-fold increase in elicitor production without significantly affecting the biomass level and the dissolved oxygen value (Figure 4). Five hours after Phe addition, the NABD concentration decreased to the same level it was before the pulse because of the dilution rate of the medium.

These results strongly suggest that Phe could be used as a precursor and/or act as a regulator in the biosynthetic pathway leading to NABD rather than as nutrient for cell growth. It is necessary to understand how phenylalanine increases NABD production. This amino acid could be a precursor of the molecule giving the cyclic core but could also play the role of amine donor.

On another hand, NABD secretion by BTP1 is probably not related to the cell-cell communication phenomenon (quorum sensing) which was found to be essential for expression of biocontrol activity by several antibiotic-producing Pseudomonas strains (van Rij et al. 2004). First, the use of three different biosensors revealed that BTP1 only produces very small amounts of N-acyl-homoserine lactone (AHL) autoinducers compared to other strains such as P. aeruginosa PAO1. Second, supplementing exponentially growing cells with concentrated supernatant extracts containing AHL did not resulted in an earlier and/or increased production of the elicitor (results not shown). Further work will be devoted to the study of other regulatory systems such as starvation or stress-related sigma factors or the GacS/GacA two-component regulatory system which is involved in the biosynthesis of a wide range of secondary metabolites or extracellular enzymes and appear to be highly conserved within the genus Pseudomonas (Haas and Keel 2003).

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References


Multitrophic interactions of *Paecilomyces lilacinus* strain 251 in the rhizosphere of host and non-host plants

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**Abstract:** The facultative egg pathogenic fungus *Paecilomyces lilacinus* is one of the most widely tested biocontrol agents for control of plant parasitic nematodes. The commercial strain 251 (PL251) is undergoing registration procedures in the EU and US and is commercially available as BIOACT® WG in several countries. To better understand the multitrophic interactions of PL251 in the rhizosphere, dose-response experiments were conducted to evaluate the relationship between the antagonist dose and biocontrol efficacy and fungal persistence. The importance of host- or non-host plants, nematodes, mutualistic fungal endophytes, and mycorrhiza for biological efficacy and unwanted side effects caused by the application of the biocontrol fungus was also investigated. It could be demonstrated that persistence and consequently the biocontrol efficacy of PL251 is not, unlike other nematophagous fungi, linked to the presence of the target nematode nor the host plant. Furthermore, some nematode host plants seem to provide unsuitable conditions in their rhizosphere resulting in rapid decline of fungal density and in some cases reduced efficacy of the antagonist. In contrast to other nematophagous fungi, rhizosphere competence is not a key factor for the biocontrol efficacy of PL251. Multiple applications did increase the persistence of the fungus in soil which was correlated with excellent control of root-knot nematodes under field conditions.

**Key words:** BIOACT®WG, root-knot nematode, biological control, risk assessment

**Introduction**

*Paecilomyces lilacinus* is a biocontrol fungus, attacking mainly eggs of root-knot and cyst nematodes. *Paecilomyces lilacinus* strain 251 (PL251) is commercially formulated and registered as a biological nematicide under the product names BIOACT® WG and MELOCON® WG in Europe and USA, respectively, and already on the market in several countries. Following application to the soil, PL251 reduces the nematode population by infecting mainly eggs and to a lesser extend juveniles and adults (Holland et al., 1999). The efficacy of PL251 has been shown in many greenhouse and field experiments (Holland et al., 2003; Kiewnick, 2004). However, still many questions remain concerning the factors and multitrophic interactions that affect the biocontrol potential of PL251.

Understanding the dynamics of a biological control agent (BCA) is important for predicting the success of biocontrol (Bidochka, 2001). Many BCAs require a certain population density to control pests or diseases (Paulitz, 2000), therefore survival of the antagonist for a certain period is important. The impact of multitrophic interactions on antagonistic fungi should be taken into consideration when biocontrol is employed. Plants exert a pronounced effect on the structure of the microbial community around the roots by the root exudates that they release into the rhizosphere (Lynch, 1990). Dose-response relationships in biocontrol systems can demonstrate the importance of inoculum density, antagonist concentration, host susceptibility to the pathogen and the proportion of a pathogen to exist within a refuge for the efficacy of the antagonist. Here, a range of attempts are presented to understand the role of multitrophic interactions in the biocontrol of plant parasitic nematodes by PL251.
Material and methods

Dose-response experiments
For all dose-response experiments, a non-sterile field soil sand mixture (1:1, v:v) was used. PL251 was applied using the glucose based water dispersible granule formulation containing at least 1 x 10^10 viable conidia per g product. Conidia, with or without formulation, were applied to soil as a suspension and incorporated using a soil mixer. In all experiments, five rates of PL251 were applied. Afterwards, nematode inoculum (1000 eggs and juveniles per 100cm³) was incorporated into the soil by hand. Soil treated with only water or water plus *M. incognita* served as controls. Pots were then covered with plastic wrap to prevent rapid drying, transferred to a growth chamber and incubated at 25 ± 1°C and a 16 hour light period. The pots were incubated for six days to allow the fungus to establish in the soil and to infect the nematode inoculum. Before transplanting, the roots of the tomato seedlings were gently washed free of soil to guarantee direct exposure to treated soil. Tomatoes were fertilized biweekly with a 20-10-15 (N-P-K) fertilizer solution. Plants were harvested 8 to 10 weeks after transplanting, and root and shoot fresh weight was determined. Nematode damage was determined by rating root galling on the 0 to 10 scale of Bridge and Page (1980) and by counting the number of egg masses per root after staining with Phloxine B (0.015%). To determine the reduction in *M. incognita* reproduction potential the number of eggs and juveniles per root system was determined using the method of Hussey and Barker (1973).

Effect of formulation and dose on persistence
Samples from PL251 treated soil were taken six days after application of conidia. Serial dilutions were prepared from 5g sub-samples and the number of colony forming units (CFU) per gram of soil was determined by plating onto OHIO-agar (Johnson and Curl, 1972). Soil samples were also taken at termination of the experiments, between 9 to 10 weeks after application to determine the number of CFU/g soil as described above. An additional greenhouse experiment was conducted to investigate the effect of repeated application (pre-, at- and post-plant) on the density of PL251 in soil. Soil samples were analyzed for the population density of PL251 on seven sampling occasions, before and after pre- and post-plant applications as described above.

Field experiment
A field experiment was conducted in Greece in 2004 following EPPO guidelines to evaluate the potential of PL251 applied as BIOACT® WG for the control of root-knot nematodes on tomato (Lüth, 2005). PL251 was applied as a soil treatment two weeks before planting. Untreated soil and Vydate (oxamyl) applied pre-planting served as controls. Seedlings received an additional drench with 1 x 10^8 conidia of PL251 per plant, 24 hours before transplanting. PL251 was applied again as a drench with the full rate of 0.2g/plant, 7 and 14 weeks after transplanting. Vydate was applied again 4 weeks after transplanting. Root-knot nematode damage was recorded 12, 18 and 20 weeks after transplanting. Cumulative fruit yield was determined at five harvest dates.

Effects of plant species
The effect of 12 plant species on the population dynamics of PL251 in bulk and rhizosphere soil was investigated in greenhouse experiments (Rumbos and Kiewnick, 2005). The decline in cfu numbers of PL251 was monitored over 100 days after soil treatment. Furthermore, for five plant species, their effect on the residual biocontrol efficacy was investigated. Plants were removed from untreated or treated soil six weeks after PL251 application and inoculated with 5000 *M. incognita* eggs and juveniles per plant. Tomato plants were then transplanted to this soil and residual biocontrol efficacy was measured by counting the galls and egg masses per root after 8 weeks in a greenhouse.
Effect of supplemental food sources
Carbon sources can affect growth and establishment of biocontrol fungi and might be used to increase efficacy. Soyamin is a selective food source rich in soy peptone and carbohydrates. To investigate the effect of Soyamin on the efficacy of PL251, soil was inoculated with 10,000 eggs and juveniles of *M. incognita* per liter and 0.1g or 0.2g product was combined with 0.01, 0.05 or 0.1g Soyamin and incorporated by hand. After 8 weeks in a greenhouse, plants were harvested, shoot and root weight determined and gallindex was evaluated using the scale of Bridge and Page (1980). In addition, roots were stained with Phloxine B (0.015%) and the number of egg masses counted. To investigate the effect of a supplemental food source on the ability of PL251 to colonize egg masses, 10 egg masses were collected from each of the six replicates, briefly surface sterilized and transferred to modified OHIO-agar (Rumbos et al. 2003). After 14 days incubation, pure cultures were transferred to potato dextrose agar identified using morphological characteristics. To investigate the effect of the supplemental food source on the populations density of PL251, soil samples were taken at the beginning and at termination of the experiment and analyzed as mentioned above.

Results and discussion

Dose-response experiments
After application of PL251 with or without glucose to soil at 5 doses, no differences in shoot or root fresh weight were found compared to both untreated or nematode inoculated controls (data not shown). Dose-response data from the experiments were analyzed using non-linear regression. The estimated parameters ED50 and EC50 for dose-response relationships with or without glucose formulation are listed in table 1. When PL251 was applied without formulation, the ED50 values for the parameters gall index, egg masses and final population per root ranged between 0.068g and 0.103g/500 cm³ soil. These rates are equivalent to a concentration (EC50) of 8.10 x 10⁵ to 1.40 x 10⁶ CFU/g soil six days after application. Similar results were obtained when PL251 was applied with the formulation resulting in ED50 values of 0.097g and 0.08g (1.29 x 10⁶ and 9.88 x 10⁵ CFU/g) for the parameters gall index and final population, respectively. However, the ED50 value for the parameter egg masses/root was more than 10 times lower (0.007g) when PL251 was applied with the formulation compared to the application without formulation. Maximum efficacy ranged between 58% and 74% depending on the parameter evaluated (Table 1).

Table 1. Dose-response relationship between pre-planting soil treatments with *Paecilomyces lilacinus* strain 251 applied with or without glucose formulation and damage of tomato roots by *Meloidogyne incognita*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PL251 without formulation</th>
<th></th>
<th>PL251 with formulation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED50 [g]</td>
<td>EC50 [cfu/g soil]</td>
<td>Efficacy [%)</td>
<td></td>
</tr>
<tr>
<td>Gallindex</td>
<td>0.068</td>
<td>8.06 x 10⁵</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Egg masses/root</td>
<td>0.096</td>
<td>1.28 x 10⁶</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Final population/root</td>
<td>0.103</td>
<td>1.40 x 10⁶</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Gallindex</td>
<td>0.097</td>
<td>1.29 x 10⁶</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Egg masses/root</td>
<td>0.007</td>
<td>2.64 x 10⁵</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Final population/root</td>
<td>0.080</td>
<td>9.88 x 10⁵</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>

*) gall index according to Bridge and Page (1980); *) g product per 500 cm³ soil; *) maximum achieved biocontrol efficacy according to Abbott (1925)
**Effect of formulation and dose on persistence**

When data on survival of PL251 in soil was analyzed for effects of the glucose formulation and the dose applied, a comparison of the slopes revealed no differences between density of conidia applied either with or without the glucose carrier (data not presented). The data of the two experiments was therefore pooled and further analyzed. Comparison of the slopes of the two curves revealed no significant differences (data not shown).

Figure 1 demonstrates the effect of multiple applications on the population dynamics of PL215 in soil. It could be demonstrated that repeated applications of the product resulted in increased densities in the soil. A single application was not sufficient to maintain the density above the threshold level needed for biocontrol (Kiewnick and Sikora, 2003). However, two or more applications with 0.2g product/plant were sufficient to establish concentrations of PL251 in the soil, similar to the EC\textsubscript{50} values obtained from dose response experiments (Table 1). These findings confirm previous studies where repeated application of PL251 significantly increased the biocontrol activity against \emph{M. incognita} on tomato (Kiewnick and Sikora, 2004).

![Figure 1](image1.png)

**Figure 1.** Effect of repeated application on the population density of \emph{Paecilomyces lilacinus} strain 251 in soil. Arrows indicate application of the biocontrol fungus with 0.2g product/plant. *) CFU numbers of other filamentous fungi in untreated and \emph{Meloidogyne incognita} inoculated soil

**Field experiment**

A trial was established to demonstrate the potential of PL251 to control root-knot nematodes under field conditions. Figure 2 shows that the biocontrol fungus demonstrated the same efficacy as the chemical nematicide either in suppressing nematode damage of roots or increase in fruit yield compared to the untreated control. Furthermore, density of \emph{M. incognita} in soil was also significantly lower compared to the control, similar to the reduction by the chemical nematicide (data not presented). Moreover, the efficacy in reducing nematode damage was about 58% when roots were rated for galling 20 weeks after transplanting. This correlates with the maximum efficacy achieved in the above mentioned dose-response experiments (Table 1).
Effects of plant species

In general, population density of PL251 in planted and non-planted soil did not increase after inoculation (Rumbos and Kiewnick, 2005). The reduction in CFU numbers compared to initial densities ranged from 72.3% to 91.3% in the first and from 80.4% to 94.9% in the second experiment. In both experiments, a linear decline of the fungal density was observed. Statistical analysis revealed that the decline in density over time was strongly negatively correlated with R² values ranging from 0.78 to 0.97 (data not shown). The decline in CFU numbers in soil planted with beans for both experiments and with sugar beet in experiment II was significantly different compared to non-planted soil (Table 2). For the remaining plant species tested, differences in decline of CFU numbers compared to non-planted soil were not significant.

Densities of PL251 in the rhizosphere in comparison to the fungal densities in soil were significantly higher in the rhizosphere of corn, barley (Exp. I) and eggplant (Exp. II) compared to non-planted soil (data not shown). Overall, it was found that in the first experiment five out of nine plant species tested showed densities in their rhizosphere that were 4 to 32% higher compared to non-planted soil. In the second experiment, seven out of ten plant species showed higher densities (3 to 68%) in their rhizosphere. In both experiments, tomato and cabbage showed lower CFU numbers in their rhizosphere compared to soil (data not shown). Comparison of slopes between the two experiments revealed significantly steeper slopes for nine out of 12 plant species and treated soil only in the second experiment (Table 2).

Concerning the importance for biological control efficacy, the most critical point is to maintain the necessary population density of the biocontrol agent long enough to protect the plant (Paulitz, 2000). Most of the plant species tested did not exert any significant effect on the dynamics of the fungus in the soil. As an exception, beans significantly affected the population density of PL251 in both experiments. The density of PL251 in rhizosphere was in most cases higher than in soil (Rumbos and Kiewnick, 2005). This effect is probably due to higher nutrient availability in the rhizosphere. In none of the experiments, PL251 was found

![Graph showing effect of BIOACT® WG and Vydate on gallindex and cumulative fruit yield on tomato under field conditions in Greece in 2004.](image-url)
to proliferate or to establish in the soil or rhizosphere. Furthermore, previous studies have shown that the decline of PL251 densities was not altered by the presence of nematode infected tomato roots (Kiewnick et al., 2004). These results indicate a behavior of PL251 in the soil and root environment which is different from other egg pathogenic fungi such as *V. chlamydosporium*. In these experiments, endophytic colonization of tomato, potato or pepper roots by PL251 was never found, and the CFU numbers in roots of banana, cabbage, corn and wheat were rather low (data not shown). However, PL251 could be isolated in high densities from barley roots. These findings are in contrast to the results of Holland et al. (2003) who did not detect hyphae of PL251 in barley roots. To our knowledge, this is the first time that PL251 is reported to endophytically colonize healthy root tissue of barley. However, further research is needed to determine whether the colonization of healthy root tissue is of advantage for biocontrol efficacy. In conclusion, the host plant is not one of the primary factors affecting the persistence of the fungus.

Table 2. Effect of 12 plant species on persistence of *Paecilomyces lilacinus* strain 251 in soil over 100 days in two greenhouse experiments

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Exp. I</th>
<th>Exp. II</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (soil only)</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>Banana</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Barley</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Beans</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cabbage</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Corn</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Cucumber</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Eggplant</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Pepper</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Tomato</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Potato</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Sugar beet</td>
<td>ns</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Wheat</td>
<td>nt</td>
<td>ns</td>
<td>nt</td>
</tr>
</tbody>
</table>

ns = non-significant; nt = not tested; *) significant different slope compared to treated soil only at \( P = 0.05 \) (Modified after Rumbos and Kiewnick, 2005)

Table 3. Effect of plant species on the residual biocontrol efficacy of *Paecilomyces lilacinus* strain 251 toward *Meloidogyne incognita* on tomato

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biocontrol effect on tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root galling</td>
</tr>
<tr>
<td>Soil only</td>
<td>…</td>
</tr>
<tr>
<td>Banana</td>
<td>+</td>
</tr>
<tr>
<td>Barley</td>
<td>-</td>
</tr>
<tr>
<td>Cabbage</td>
<td>-</td>
</tr>
<tr>
<td>Eggplant</td>
<td>○</td>
</tr>
<tr>
<td>Corn</td>
<td>○</td>
</tr>
</tbody>
</table>

+ = biocontrol activity enhanced compared to treated soil only; ○ = same as soil only; - = negative effect
In addition, it was demonstrated for the first time that a host plant can affect the residual biocontrol efficacy of PL251 in soil (Table 3). Soil in which banana plantlets had been grown for 5 to 6 weeks seemed to favor the activity of PL251 against root-knot nematodes. So far, this effect has been described for rhizosphere competent fungi such as *Pochonia chlamydosporia*. For this fungus Kerry and Hidalgo-Diaz (2004) developed an integrated control system based on the beneficial effects of nematode host and non-host plants which significantly affected establishment and biocontrol activity *P. chlamydosporia*.

Table 4. Effect of *Paecilomyces lilacinus* strain 251 alone or in combination with Soyamin on root-knot nematode damage, egg mass colonization on tomato and fungal density in soil

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gallindex&lt;sup&gt;x&lt;/sup&gt;</th>
<th>egg mass/</th>
<th>% colonized</th>
<th>log CFU/g soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>root</td>
<td>egg masses&lt;sup&gt;y&lt;/sup&gt;</td>
<td>0 dpa&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control, untreated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. incognita</em></td>
<td>4.33 a</td>
<td>72.7 a</td>
<td>0.0</td>
<td>6.33 a</td>
</tr>
<tr>
<td>0.1g PL251&lt;sup&gt;v&lt;/sup&gt;</td>
<td>4.50 a</td>
<td>46.2 bc</td>
<td>30.0</td>
<td>5.79 b</td>
</tr>
<tr>
<td>0.1g PL251 + 0.01g SOY&lt;sup&gt;w&lt;/sup&gt;</td>
<td>3.17 bc</td>
<td>58.2 ab</td>
<td>32.5</td>
<td>5.99 ab</td>
</tr>
<tr>
<td>0.1g PL251 + 0.05g SOY</td>
<td>4.33 a</td>
<td>54.2 ab</td>
<td>30.0</td>
<td>6.08 ab</td>
</tr>
<tr>
<td>0.1g PL251 + 0.10g SOY</td>
<td>4.50 a</td>
<td>49.7 bc</td>
<td>32.5</td>
<td>6.40 a</td>
</tr>
<tr>
<td>0.2g PL251 + 0.01g SOY</td>
<td>2.33 c</td>
<td>29.2 c</td>
<td>20.0</td>
<td>6.32 a</td>
</tr>
<tr>
<td>0.2g PL251 + 0.05g SOY</td>
<td>3.67 ab</td>
<td>54.3 ab</td>
<td>26.0</td>
<td>6.40 a</td>
</tr>
<tr>
<td>0.2g PL251 + 0.10g SOY</td>
<td>3.33 b</td>
<td>41.8 bc</td>
<td>29.2</td>
<td>6.26 a</td>
</tr>
</tbody>
</table>

Means in one column followed by the same letter are not significantly different at $P = 0.05$ (n=6);<sup>v</sup>) dose of PL251 in g product/liter soil, one g contains $1 \times 10^{10}$ viable conidia;<sup>w</sup>) dose of Soyamin in g/liter soil;<sup>x</sup>) Gallindex according to Bridge and Page (1980);<sup>y</sup>) egg masses colonized with *Paecilomyces lilacinus* strain 251;<sup>z</sup>) days past application; ns = non-significant.

**Effect of supplemental food sources**

The initial dose-response experiments conducted with and without the glucose formulation might lead to the conclusion that in the presence of the glucose, ED<sub>50</sub> values are lower. Glucose or other supplemental nutrients can affect chlamydospore or conidia germination and survival of biocontrol agents and may influence biocontrol efficacy. In the case of PL251, it has been demonstrated that germ tube elongation was more rapid on media rich in sugars compared to water agar (Kiewnick, 2004). This might have resulted in a more rapid reduction of the nematode inoculum. Similar effects were observed when a protein rich food base was added to soil together with the biocontrol agent (Table 4). At a concentration of 0.01g Soyamin/liter soil, biocontrol efficacy of PL251 was clearly increased. However, these increases in biocontrol efficacy neither correlated with the potential of PL251 to colonize egg masses of *M. incognita* nor with the density of the fungus in soil at the termination of the experiment. This leads to the conclusion that the initial reduction of the inoculum is mainly affected by the density of PL251 in soil, the speed of conidia germination and infection of nematode eggs.

**Multitrophic interactions**

As demonstrated by the present data, rhizosphere competence is not a key factor for the biocontrol efficacy of PL251. This seems a favorable feature of this biocontrol fungus, because it makes it more suitable for combinations with other antagonists mitigating the risk
of unwanted adverse side effects. Preliminary studies on the interactions between PL251 and either mutualistic fungal endophytes or mycorrhizal fungi revealed no side effects on these antagonists after application of high doses of the commercial product (Rumbos et al., 2005). Besides a distinct additive effect in the biological control of root-knot nematodes on tomato, no reduction in the colonization of the roots by fungal endophytes or mycorrhizal fungi was found.

In conclusion, the fungal egg pathogen *Paecilomyces lilacinus* strain 251 is an excellent biocontrol agent against plant parasitic nematodes on a variety of crops. As demonstrated, reduction of inoculum in soil rather than colonization of the rhizosphere is important for the success in reducing nematode damage. Although rhizosphere competence seems not a key factor, further research is needed to fully evaluate the multitrophic interactions with plants, target pest and other antagonists in soil or in the rhizosphere of plants.

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Commercial research and development of disease-suppressive microorganisms

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Key words: biopesticide, registration, commercial development, biocontrol

Introduction

Research over the past 40 years has yielded numerous contributions to our understanding of the mechanisms and microorganisms responsible for biologically mediated plant-disease suppression. Despite the intense and widespread effort and tremendous public and private resources invested in achieving these results, few commercially viable and dependable biologically mediated plant disease suppression strategies are widely used by growers worldwide. Though the failure of translating basic science into practical technology is often blamed for this shortcoming, several other factors are more likely to affect development and implementation of effective and reliable biological disease suppression products and practices.

The discovery, characterization, primary application development, and accumulation of basic technical knowledge of disease-suppressive microorganisms (DSMs) by institutional researchers are crucial to industrial development of DSM products. Many researchers involve industry partners directly in their projects, namely as members of user committees, financial supporters, and applied research cooperators, with the intent to commercialize promising DSM products. Companies that pursue further research and development of these DSMs must confront tremendous risks and daunting challenges before products can be marketed. Properly anticipating and addressing the challenges while carefully calculating risks certainly help companies to navigate their positions clear of major potential pitfalls. From our recent experiences with EU-level biopesticide registration and our perspective between university research and commercial product development, we present several key considerations when attempting to bring a DSM product to the market today.

Three domains of development of disease-suppressive microorganisms

Those researchers and companies interested or engaged in developing biopesticide products should approach this process from different angles and consider how multidimensional the process can be. We have devised Science, Practice, and Approval as three domains or classifications of items we have encountered in our DSM commercialization activities (Figure 1).

Science
This is the domain from which the process begins. Research in the field of biological disease suppression is motivated by the increasing need for alternatives to managing plant diseases with synthetic pesticides or rapid genetic modification. In most cases, DSMs are isolated from systems or ecological niches in which disease suppression is observed and are
determined with bioassays to be suppressive to pathogens, diseases, or both. Once suppressiveness of the DSM is established, researchers frequently define, verify and report the mechanism(s) involved in the suppressive effect.

Figure 1. The domains of development of disease-suppressive microorganism products

Further characterization of the DSM and its suppressiveness should then be conducted. This is not only scientifically important, but is critical for commercial and regulatory reasons (under the Practical and Approval domains, respectively). Initially, the spectrum and extent of disease suppression and ranges of hosts and environments where DSM-mediated suppression occurs should be evaluated as early as possible to: 1) identify potential crops, cropping systems, and circumstances where the DSM product prevents or restricts disease; 2) establish limits for effective DSM use based on application timing, pathogen thresholds, and realistic DSM rates; and 3) define physical, chemical and cultural factor extremes that conflict with DSM establishment and efficacy. Finally, full characterization of toxins and metabolites produced by the DSM and its relatedness to detrimental microorganisms in close taxonomic proximity should be completed before pursuing commercialization. These additional studies are essential for early business plan or market development, and may be applied to meet some of the biopesticide registration data requirements, especially if these studies are supervised by university laboratories or GLP/GEP-certified organizations.

**Practice**

Items in this domain relate to the actual commercial development aspects that should be addressed by the entity seeking to commercialize the DSM. Practice is a mix of both business and applied science considerations constituting the following five categories:

1. Potential markets: focuses on a product positioning and market analysis that must be completed before engaging in major commercial investment and activity. Considerations in this category should include:
   - cropping systems, market niches and target customers
   - market area
   - marketing or distribution system
   - cost the customer is willing to bear (including direct cost of product and indirect cost associated with other inputs related to product use)
   - competition
   - duration of the product on the market.

2. Applied research and development: represents the compiling of in-depth technical characteristics of the DSM product and development of the actual biopesticide product by:
   - defining the suppressive system (i.e. pathosystem and type of culture being considered for application)
• extensive examination of DSM product efficacy under diverse practical conditions to help identify the technical limits of the DSM
• development, testing and determination of DSM product formulation, production process, packaging, storage conditions, shelf life, and other product characteristics
• establishing compatibility profile of DSM product use with other crop production inputs (e.g., other biopesticides, synthetic fungicides, specialty products, etc.)
• careful monitoring and proper justification of research and development costs.

3. DSM product: encompasses capabilities, costs and inherent responsibilities of producing and marketing a biopesticide that a company must assume. Firms must develop commercialization plans that properly anticipate:
   • production capacity and costs
   • quality assurance
   • marketing and distribution costs
   • registration or other government approval
   • product liability

4. Return on investment: the factor that ultimately determines whether a company pursues and continues commercialization of a DSM product. Projected sales over a specified time must be able to generate a profitable return to investors once the following costs have been recovered:
   • DSM license or complete technology acquisition costs
   • applied research and development costs
   • government approval or registration costs (i.e. application costs and costs associated additional government-directed studies necessary to complete registration dossier)
   • production, marketing, and distribution costs
   • legal representation.

Based on current EU registration requirements dictated by the latest OECD guidelines, we estimate the cost of registering and commercializing a new biopesticide to be € 6-8 million over the course of 6-10 years. Therefore, strong financial incentives must exist to interest companies in commercially developing new biopesticide products.

5. Contribution to development of future products: though not necessary to consider for commercialization of a single DSM product, this point should be considered if a company hopes to offer other biopesticide products that meet other pest suppression needs in the industry and to provide biopesticide products for many years. Some profit may be planned to be set aside for new DSM acquisition, applied research and development costs, government approval or registration, and initial production and marketing expenses.

Approval
Government legislation, regulation and acceptance ultimately influence the fate of DSM commercialization. The type of government approval depends on the nature of the product claims and whether national and local governments have designations of disease-suppressive products that do not require full biopesticide registration (i.e. registered biopesticide vs. plant strengthener, soil improver, etc.). The level of government approval depends on the intended market area (i.e. in all EU member states or selected countries).

For EU-wide biopesticide registration, a rapporteur nation must be selected to submit the registration dossier. Selecting the home country of the applicant is not required. The rapporteur nation should be chosen on the basis of: 1) the time required for the registration authority of a country to process and submit the dossier; 2) the levels of organization, experience, and preparedness of the registration authority; 3) the resources and advice available from the registration authority; 4) registration application costs; and 5) government-
backed incentives for biopesticides registration. Documented experience of the registration authority and rapid application are the most important criteria to consider to promote quicker review with fewer surprises, accelerating entry of the product on the market as soon as possible. Once a dossier is accepted by the registration authority, temporary permission to sell the product in that country can be granted. Upon receiving this permission by the rapporteur country, the applicant may seek temporary permits to sell its biopesticide in other countries, provided that it meets any additional criteria and administrative fees are paid.

EU biopesticide registration requirements focus on safety, efficacy and characterization of the DSM. The safety portion of the dossier seeks various toxicology, environmental toxicology and fate, and non-target effects data. Compulsory efficacy data must demonstrate the intended benefits of using the DSM and indicate what level of efficacy can be achieved in representative bioassays. DSM characterization requirements include establishing taxonomic position, traceability, metabolite and toxin production profiles, and mechanism(s) of suppressive activity of the DSM. All studies conducted to fulfill these requirements must be supervised by independent GLP- and GEP-certified facilities.

Discussion

The considerations and perspectives presented above are intended to guide those contemplating marketing of biopesticides through the DSM commercialization process. Many of the difficulties and disappointments that people face during this process can be avoided by keeping this information in mind. Two additional pieces of advice may also be helpful when considering DSM commercialization:
1) start first from the regulatory vantage point; and
2) hire a company to help that is knowledgeable about and experienced in pesticide registration.

References

Enrichment and genotypic diversity of \textit{phID}-containing fluorescent \textit{Pseudomonas} spp. associated with crop monoculture

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Abstract: Fluorescent \textit{Pseudomonas} spp. producing the antibiotic 2,4-diacyltphloroglucinol (2,4-DAPG) play a key role in take-all decline, which develops when a field is continuously cropped to wheat or barley after a take-all outbreak, and also contribute to the disease suppressiveness of other soils. In this study, we showed that over many years of monoculture, the crop species grown in a field enriches for genotypes of 2,4-DAPG producers from the reservoir of genotypes naturally present in the soil that are especially adapted to colonize the rhizosphere of the crop grown.

Key words: Antibiotic-producing bacteria, Host preference, 2,4-Diacetylphloroglucinol (DAPG).

Introduction

Isolates of fluorescent \textit{Pseudomonas} spp. producing the antibiotic 2,4-DAPG are responsible for some of the best examples of natural microbial defense of plant roots (Weller et al., 2002). For example, in both Washington State and Dutch fields, 2,4-DAPG producers play a key role in take-all decline (TAD), a natural suppression of take-all developing when a field is continuously cropped to wheat or barley following a severe outbreak of the disease. However, to be effective against take-all, the population of 2,4-DAPG producers must reach a threshold density of 10\textsuperscript{5} CFU/g root (Raaijmakers and Weller, 2001; Weller et al., 2002). 2,4-DAPG producers also contributes to the suppressiveness of certain soils in Switzerland to black root rot of tobacco caused by \textit{Thielaviopsis basicola} (Weller et al., 2002). In addition, evidence is mounting that monoculture of other crops results in an enrichment of 2,4-DAPG producers.

In this work we present results from different studies that support the hypothesis that crop monoculture enriches for 2,4-DAPG producers and that the crop species grown modulates the genotypes enriched from those present in the soil.

Material and methods

\textit{Soils and plants}

Crop monoculture and non-monoculture soils from the U.S.A. used in the study are listed in Table 1. Soils included an unique site on the campus of North Dakota State University, Fargo, ND, where side-by-side fields have undergone continuous wheat, continuous flax or crop rotation for over 100 years. A soil from a field located at the Washington State University Research and Extension Center, Mount Vernon, WA, that is suppressive to Fusarium wilt of pea, and had been cropped for over 30 continuous years to pea varieties as a
part of a breeding program. Also, an agricultural soil from Quincy, WA, designated Quincy take-all decline (TAD) soil that is suppressive to take-all of wheat and had been cropped to wheat for over 25 years was used. Flax (*Linum usitatissimum* L.) cv. Norlin, Pea cvs. Little Marvell and Quincy, and the spring wheat (*Triticum aestivum* L.) cv. Penawawa were used as trap plants (Landa et al., 2002; 2003; Raaijmakers and Weller, 2001).

**Determination of population densities of 2,4-DAPG producers in soil**

Different cycling experiments conducted as described before (Landa et al., 2002; 2003; Raaijmakers and Weller, 2001) were performed to quantify the population sizes of 2,4-DAPG producers that developed in the rhizosphere of flax, pea or wheat, and to collect isolates for studies of genetic diversity within the populations of these soils. Also, cycling experiments were used to determine the rhizosphere competence of selected genotypes of 2,4-DAPG producers on each crop. Population densities were determined by two methods: colony hybridization or by a PCR-based dilution endpoint method as described previously (Raaijmakers et al., 1997, Landa et al., 2002).

**Genotypic fingerprinting of indigenous phlD+ Pseudomonas spp.**

A whole-cell rep-PCR fingerprinting method with the BOXA1R primer (BOX-PCR) and RFLP analyses of *phlD*, a gene which functions in the synthesis of the 2,4-DAPG precursor monoacetylphloroglucinol (MAPG), with *Hae*III, *Msp*I and *Taq*I restriction enzymes were used to determine the genotype of over 900 2,4-DAPG-producing fluorescent *Pseudomonas* isolates among the different studies. BOX-PCR, RFLP-PCR analysis, gel electrophoresis, and assignation of an isolate into a genotype were performed as described previously (Landa et al., 2002; McSpadden et al., 2000).

**Results and discussion**

**Population densities and genotypes of indigenous 2,4-DAPG-producers**

The population dynamics of indigenous 2,4-DAPG producers in the different monoculture soils used in the study reached and sustained threshold population sizes higher than log 5.0 CFU/g root, known to be required for disease suppression (Table 1) (Raaijmakers and Weller, 2001; Weller et al., 2002). From the different isolates obtained from the soils analyzed (Table 1) and those from a collection we have identified 18 genotypes (denominated A to Q, and T) that can be differentiated using both BOX-PCR and RFLP analysis of *phlD* (Figure 1).

**Genotypes of 2,4-DAPG-producers differ in ability to colonize the rhizosphere of different crops**

With this study, we demonstrated that the genetic profiles generated by BOX-PCR and RFLP- *phlD* analysis are predictive of the rhizosphere competence of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. in the flax, pea and wheat rhizospheres, and now think that isolates of some genotypes preferentially colonize the roots of certain crop species. This idea is supported by findings that D-genotype isolates are dominant in the rhizosphere of wheat grown in Washington State TAD soils even though three other genotypes (B, L, and E) also are found in these soils (Table 1; Landa et al., 2002; McSpadden et al., 2000). Similarly, in the rhizosphere of pea grown in Mt. Vernon pea monoculture soil, genotype D- and P-isolates are dominant even though A-, L-, O-, and Q-isolates also are found in the soil (Table 1; Landa et al., 2002). Furthermore, we showed that flax monoculture, like wheat monoculture, enriches for 2,4-DAPG producers in the rhizosphere, but the composition of the genotypes enriched by the two crops is quite different (Table 1). In the Experiment using three Fargo soils, D-genotype isolates were dominant in the wheat monoculture soil but monoculture of flax enriched for F- and J-genotype isolates. On the contrary, only J-genotype isolates were detected among those recovered from the Fargo soil under continuous rotation, with population smaller than log 5.0 CFU/g root.
Figure 1. Genotypes of 2,4-diacetylphloroglucinol producing *Pseudomonas* spp. determined by restriction fragment length polymorphism (RFLP) analysis of *phlD* sequences amplified with B2BF and BPR4, and digested with *Hae*III, *Taq*I, or *Msp*I. RFLPs detected by these three enzymes were sufficient to distinguish all 18 genotypes previously defined (A to Q, & T). A 100-bp DNA size standard (*) indicates the size of the amplified products.

Table 1. Soils under monoculture used in the study and population densities and genotypes of 2,4-diacetylphloroglucinol producing *Pseudomonas* spp. that were selected by each trap plant. Underline genotypes were dominant in the rhizosphere of the trap plant grown in each soil.

<table>
<thead>
<tr>
<th>Location/trap plant</th>
<th>History</th>
<th>Inoculum density of <em>phlD</em> &gt; 10⁵ CFU/g of root</th>
<th><em>phlD</em>⁺ Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quincy/wheat &amp; pea</td>
<td>&gt;25 years continuous wheat</td>
<td>+</td>
<td>B, D, E</td>
<td>Landa et al., 2002; 2003 McSpadden et al., 2000</td>
</tr>
<tr>
<td>Fargo wheat</td>
<td>&gt;100 years continuous wheat</td>
<td>+</td>
<td>D, I</td>
<td>Landa et al., unpublished McSpadden et al., 2000</td>
</tr>
<tr>
<td>Fargo wheat</td>
<td>&gt;100 years continuous flax</td>
<td>+</td>
<td>D, E, G, J, &amp; T</td>
<td>Landa et al., unpublished McSpadden et al., 2000</td>
</tr>
<tr>
<td>Fargo wheat</td>
<td>&gt;100 years continuous rotation</td>
<td>–</td>
<td>J</td>
<td>Landa et al., unpublished</td>
</tr>
<tr>
<td>Mt. Vernon/pea</td>
<td>&gt;30 years continuous pea</td>
<td>+</td>
<td>A, B, D, L, O, P, &amp; Q</td>
<td>Landa et al., 2002</td>
</tr>
</tbody>
</table>
The above findings support our hypothesis that genotypes of 2,4-DAPG producers differ in their preference for colonizing the rhizosphere of a particular crop. Thus, introduced isolates of genotype D, and genotypes D and P, colonized the rhizosphere of wheat (Landa et al., 2003) and pea (Landa et al., 2002), respectively, significantly better than the other genotypes that were not dominant in the monoculture soils where the genotypes were originally isolated. We also showed that isolates belonging to genotypes D and P, or genotypes D and K, or J, are significantly more aggressive at colonizing the rhizosphere of pea, wheat, and flax, respectively than isolates of other genotypes (Landa et al., 2002; 2003; unpublished). Furthermore, genetic profiles generated by rep-PCR or RFLP analysis of phlD were shown to be predictive of the rhizosphere competence of introduced 2,4-DAPG-producing strains on each of the crop analyzed (Figure 1).

Our results showing variation in the ability of different genotypes of 2,4-DAPG producers to colonize the root of different crops (flax, pea and wheat) suggest that specific genes in the bacteria play a role complementary to those in the plant supporting this interaction. A major challenge for the future is the identification of the genes in those genotypes preferentially enriched by a crop and the genes in the host plant that govern such remarkably efficient root colonization.

Acknowledgements

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References


Combining *Brassicaceae* green manure with *Trichoderma* seed treatment against damping-off in sugar beet

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**Abstract:** In recent years different biological approaches of varying degrees of efficacy have been developed with the aim of controlling plant diseases. One of these is represented by the green manure of *Brassicaceae* plants, which provides a natural fumigation based on the defensive myrosinase-glucosinolate system. After tissue damage, glucosinolate enzyme-derived compounds are released with a toxic effect on phytopathogenic fungi. Another approach is represented by the utilisation of biological control agents, like the soil saprophytic fungi *Trichoderma* spp. which was shown to be effective against several soil-borne diseases through different mechanisms. The aim of this work was to evaluate the combined effects of biofumigation and *Trichoderma* treatment on the control of damping-off in sugar beet.

A commercial mixture of *Brassica napus* varieties selected against different soil pathogenic fungi was sown at the dose of 10 kg ha$^{-1}$ on a soil highly infested by *Pythium* and *Rhizoctonia* in central Italy. Sixty days after growing, an estimated biomass of 67 t ha$^{-1}$ of green material was incorporated into the soil by a rotating harrow at 20 cm depth. After one month samples of amended and unamended soil were collected and utilised for an experiment in greenhouse to evaluate the protective effect against sugar beet damping-off, in a randomised block design with six replicates (pots), twenty seeds per pot. The effect of two different *Trichoderma* strains applied to sugar beet seeds was evaluated in comparison to untreated seeds both on amended and unamended soil. Such strains were previously selected for *in vitro* activity against *Pythium* spp. and *Rhizoctonia* spp.

Factorial ANOVA analysis highlighted a statistically significant effect of the green manuring on the number of healthy plants as well as of *Trichoderma* seed treatment, which showed an adjunctive protective effect after *Brassica napus* green manuring.

**Key words:** *Brassicaceae*, green manure, *Trichoderma*, seed treatment, sugar beet, damping-off

**Introduction**

In recent years an increasing demand has developed for sustainable agricultural systems aimed at safeguarding the environment. Concerning disease management, several methods of control have been studied to reduce chemical inputs into the environment, with different levels of efficacy. In particular soil-borne pathogens appear to be among the most difficult to control, even by chemicals, probably due to the complicated ecosystem of the soil, where a number of interactions occur. The adoption of correct agronomic practices normally prevents damage, but in the presence of favourable conditions the disease rapidly spreads almost without any possibility of control, apart from methods with a high environmental impact like soil fumigations. Softer alternatives are represented by biological methods but the awareness of their moderate effectiveness suggests combining some of them in a multiple integrated approach.

In this light methods aimed at lowering pathogen inoculum in soil could be performed at different levels: a) directly, by the green manure of biocidal crops, like *Brassicaceae*, which are known to provide a natural fumigating effect by releasing fungitoxic compounds;
b) indirectly, by treating the crop seeds with Trichoderma strains selected for rhizosphere competence and antagonism towards the considered pathogens.

Biocidal crops contain secondary metabolites, glucosinolates, which play a defensive role together with the endogenous myrosinase enzyme. After tissue damage, glucosinolate enzyme-derived compounds, mainly isothiocyanates, are released with a toxic effect on several pathogens (Brown & Morra, 1997). For centuries a general phenomenon of noxious organism suppression had been observed after *Brassicaceae* cultivation or incorporation into the soil, but it has recently received renewed interest, due to the need to find alternative options to chemical fumigants. The term of biofumigation was therefore coined, since volatile toxic compounds are released into the soil by the green manure technique, and a number of experiments were conducted against several soil pathogens, with varying degrees of efficacy (Kirkegaard & Matthiessen, 2004).

*Trichoderma* spp. are saprophytic soil fungi known as biological control agents (BCA) active against several plant pathogens. They have proved to be effective in a large number of crops against soil-borne and seed-borne diseases, storage rots and diseases in the phyllosphere, acting through different mechanisms such as competition, antibiosis, mycoparasitism, enzyme production, induction of defence responses and other adjunct mechanisms, such as growth promotion (Howell, 2003). Moreover, the ability to readily colonise the infection court is an important pre-requisite together with the replacement of the endogenous microflora (Harman, 2000). *Trichoderma* spp. preferentially grow within the soil on plant debris, rather than on healthy tissues, unless a specific competence has been developed, is also obtainable through mutation and selection (Ahmad & Baker, 1987).

The aim of this study was to evaluate the combined effects of biofumigation and *Trichoderma* seed treatment on the control of damping-off in sugar beet.

**Material and methods**

A commercial mixture of *Brassica napus* varieties (BQ Mulch, S.I.S., Italy), selected against different soil pathogenic fungi, was sown at the dose of 10 kg ha$^{-1}$ on a soil highly infested by *Pythium* and *Rhizoctonia* at Pontinia (LT) in central Italy, at the beginning of August 2004. The experimental scheme was a randomised block design with 2 replicates, plots of 540 m$^2$, including untreated plots as controls. Sixty days after growing (October 2004), an estimated biomass of 67 t ha$^{-1}$ of green material was incorporated into the soil by a rotating harrow at 20 cm depth.

**Greenhouse experiment**

One month after (November 2004) samples of amended and unamended soil collected from the field were transferred in the greenhouse to evaluate both the effect of biofumigation and of BCA against sugar beet damping-off. Sugar beet seeds (cv Semper) were treated by two different *Trichoderma* strains (S2 and B41), applied as homogenate of liquid culture. These strains were previously selected for their *in vitro* antagonistic activity against different soil-borne pathogens, measuring the colony growth inhibition (%) after 48h at room temperature on PDA poisoned by 25% liquid culture filtrate of different *Trichoderma* strains. Treated and untreated sugar beet seeds were sown both on amended and unamended soil in a randomised block design with six replicates (pots) and twenty seeds per pot.

**Field experiment**

In November 2004 untreated and *Trichoderma* (S2 and B41) treated seeds of sugar beet (cv Semper) were sown (15 seeds m$^{-2}$) both on amended and unamended plots in a split-plot design with 2 replicates and 2 factors: amended/unamended on main plots; *Trichoderma* treated/untreated sugar beet seeds on sub-plots. The number of healthy sugar beet plants per m$^2$ was recorded in spring 2005.
Results and discussion

Table 1 reports the results of the in vitro assay which led to the selection of the two *Trichoderma* strains, B41 being the most active against *Phytophthora* and S2 being the best against *Rhizoctonia*.

Table 1. Colony growth inhibition (% of different pathogens after 48 h at room temperature on PDA poisoned by 25% liquid culture filtrate of different *Trichoderma* strains).

<table>
<thead>
<tr>
<th>Trichoderma</th>
<th>Colony growth inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Phytophthora</em></td>
</tr>
<tr>
<td>B41</td>
<td>94.4 a</td>
</tr>
<tr>
<td>S2</td>
<td>55.2 b</td>
</tr>
<tr>
<td>Lt31</td>
<td>45.6 c</td>
</tr>
<tr>
<td>Lt33</td>
<td>10.4 d</td>
</tr>
<tr>
<td>Lt35</td>
<td>0.0 e</td>
</tr>
<tr>
<td>Lt36</td>
<td>0.0 e</td>
</tr>
</tbody>
</table>

Values in columns followed by the same letter are not different for $P \leq 0.05$ (L.s.d. test after arcsin transformation).

**Greenhouse experiment**

Green manuring, as well as *Trichoderma* seed treatment, S2 strain especially, significantly increased the number of healthy plants (fig. 1). *Trichoderma* treatment also showed an adjunctive protective effect on amended soil (fig. 2).

![Graph showing sugar beet healthy plants](image)

Figure 1. Green manure and *Trichoderma* effects on sugar beet damping-off in greenhouse (mean values). Bars with the same letter are not different for $P \leq 0.05$ (L.s.d. test after arcsin transformation).
Figure 2. Adjunctive effect of seed treatment by B41 and S2 *Trichoderma* strains on amended and unamended soil. C is the untreated control.

**Field experiment**
*Brassicaceae* green manure made it possible to obtain a final plant density of sugar beet significantly higher than the control, while *Trichoderma* treatment was ineffective both on amended and unamended soil (fig. 3).

Figure 3. Effect of green manure and *Trichoderma* seed treatment on sugar beet plant density in open field (mean values). Bars with the same letter are not different for P≤0.05 (l.s.d. test after arcsin transformation).

In conclusion, combining *Brassicaceae* green manure with *Trichoderma* seed treatment showed interesting effects on sugar beet damping-off under controlled conditions. In open field *biofumigation* maintained effectiveness while *Trichoderma* treatment was ineffective probably due to adverse environmental factors.

**References**


Mycotoxin modulation of chitinase gene expression in a plant-beneficial fungus used for biological control

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2 Agroscope FAW Wädenswil, CH-8820 Wädenswil, Switzerland

Abstract: Biocontrol agents such as *Trichoderma* were applied in many different environments. These habitats were potentially also occupied by mycotoxin producing fungi. This indicates that *Trichoderma* will likely encounter an array of mycotoxins. But little is known how these fungal toxins affect biocontrol efficacies. Any competitive advantage conferred by mycotoxins would complicate efforts to control mycotoxigenic fungi. We examined the influence of important mycotoxins on expression of *Trichoderma* chitinases using *ech42-goxA* or *nag1-goxA* reporter gene fusions in *T. atroviride* P1. Production of chitinases, such as the ECH 42 endochitinase and the N-acetyl-β-glucosaminidase NAG1, is a primary mechanism of action for *T. atroviride* P1. Recently, the molecular interaction between the *Fusarium* mycotoxin deoxynivalenol (DON) was evaluated in detail. It was found that DON down-regulates *nag1* gene expression in the fungal antagonist *Trichoderma atroviride* P1. Using a broader approach, additional mycotoxins that are prevalent in various crops and environments were evaluated in vitro for their ability to interact with the antagonistic fungus *Trichoderma atroviride* strain P1. Three different patterns were identified with certain mycotoxins having no impact on expression of either chitinase gene (patulin, fumonisin B1, and beauvericin); others reduced significantly *nag1*, but not *ech42* expression (DON, ochratoxin A), and a third group induced *ech42*, but had no influence on *nag1* expression in *T. atroviride* P1 (aflatoxin and α-zearalenol). As sole mycotoxin beauvericin reduced significantly *Trichoderma* growth. Implications for the understanding of ecological functions of mycotoxin production and for deployment of biocontrol strategies are discussed.

Key words: *Trichoderma*, mycotoxins, gene expression, chitinases

Introduction

Mycotoxins produced by *Aspergillus, Fusarium, Penicillium* and other fungi have broad-spectrum toxicity to humans and other animals, plants and microorganisms at low concentrations (Bennett and Klich 2003; D’Mello and Macdonald 1997; Desjardins and Hohn 1997; Keyser et al. 1999). These fungal toxins are a global concern with over one-quarter of the world food-and-feed crop contaminated annually. In the United States alone, conservative estimates place economic losses from contaminated crops and efforts to control and monitor contamination at $500 million to $1.5 billion (Cardwell et al. 2001; CAST 2003; Robens and Cardwell 2003).

Biological control using introduced microbial antagonists is a promising approach for sustainable reduction of crop infection and contamination. Atoxigenic strains (Bock and Cotty 1999), yeasts (Khan et al. 2004) and *Trichoderma* (Lutz et al. 2003), which are able to aggressively colonize infection courts and survival habitats of mycotoxigenic fungi are potential biocontrol agents. These antagonists of mycotoxigenic fungi will be confronted with pathogen toxins, and will likely encounter an array of toxins in the different environments where they will be deployed. But the performance of antagonists can be compromised by...
negative interactions with certain pathogen toxins (Duffy et al. 2003). Thus, it is imperative that we understand antagonist interactions with a wide range of mycotoxins. Much of our understanding of such interactions deals with toxin-mediated gene repression in bacterial antagonists (Duffy and Défago 1997; Duffy et al. 2004; Notz et al. 2002; Schnider-Keel et al. 2000). Recently, we found that one mycotoxin, deoxynivalenol (DON), down-regulates nag1 gene expression in the fungal antagonist *Trichoderma atroviride* P1 by using three approaches (i.e., application of purified toxin, co-inoculation with natural producing and non-producing strains, co-inoculation with insertion mutants lacking DON production) *in vitro* as well as on maize residues (Lutz et al. 2003). The nag1 gene encodes for one of two chitinases that are critical to the primary mechanism of action, mycoparasitism (Brunner et al. 2003; Lorito 1998; Woo et al. 1999). The mechanism behind this negative interaction is still unknown.

The type of toxigenic fungi and/or toxins that may be present to potentially interfere with biocontrol, however, depends largely upon environmental factors (climatic, host genotypic, etc.). In this study, we have taken a broad approach examining interactions *in vitro* of *T. atroviride* P1 with several mycotoxins representative of those that could be encountered in the diverse environments where *Trichoderma* is potentially applied.

### Material and methods

Derivatives of *T. atroviride* strain P1 carrying goxA-reporter gene fusions with the *ech42* (derivative *ech42-gox*) or the *nag1* (derivative *nag1-gox*) chitinase gene, which have previously been described (Mach et al. 1999), were used to sensitively monitor chitinase gene expression as in other studies (Lutz et al., 2003, Lutz et al., 2004). *Trichoderma* strains were stored as spore suspensions in 20 % glycerol at -20°C and grown for each experiment on 1.5 % malt extract agar (MA) plates for 4 days at 24°C in darkness. Mycotoxins dissolved in methanol (0.4%) were added to MA and controls consisted of the same amount of methanol (Table 1).

### Table 1. Mycotoxins tested

<table>
<thead>
<tr>
<th>Mycotoxin a</th>
<th>Fungal producer</th>
<th>Tolerable levels b Media concentration c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B1</td>
<td><em>Aspergillus</em></td>
<td>0.002 (µg g⁻¹) 0.004 (µg g⁻¹)</td>
</tr>
<tr>
<td>Beauvericin</td>
<td><em>Fusarium</em></td>
<td>n.a. (µg g⁻¹) 2.000 (µg g⁻¹)</td>
</tr>
<tr>
<td>Fumonisin B1</td>
<td><em>Fusarium</em></td>
<td>1.000 (µg g⁻¹) 2.000 (µg g⁻¹)</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td><em>Aspergillus</em></td>
<td>0.005 (µg g⁻¹) 0.010 (µg g⁻¹)</td>
</tr>
<tr>
<td>Patulin</td>
<td><em>Penicillium</em></td>
<td>0.050 (µg g⁻¹) 0.100 (µg g⁻¹)</td>
</tr>
<tr>
<td>α-Zearalenol</td>
<td><em>Fusarium</em></td>
<td>n.a. (µg g⁻¹) 0.040 (µg g⁻¹)</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td><em>Fusarium</em></td>
<td>1.000 (µg g⁻¹) 2.500 (µg g⁻¹)</td>
</tr>
</tbody>
</table>

a Mycotoxins were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

b Tolerable levels according to the Swiss Federal Law on Foodstuffs (Fremd- and Inhaltsstoffverordnung SR817.0), Ordinance on Contaminants (SR817.021.23) harmonized with EU standards; n.a. indicates level not set.

c Final concentration of the mycotoxin in amended media.

d Experiment was done in Lutz et al., 2003

One plug of an actively growing culture of *T. atroviride* P1 reporter strain *ech42-gox* or *nag1-gox* was placed inverted in the centre of a plate and incubated for 66 h at 24°C in darkness.
Trichoderma growth and GOX activity was assessed as described in Lutz et al. (2003). GOX levels were quantified in the supernatant according to Mach et al. (1999). Each experiment was conducted three times with four replicates in each trial. Data were pooled after determining there was no significant trial x treatment interaction using Systat version 9.0 (Systat Inc., Evanston, IL). Mycotoxin treatments were compared with controls using t-tests (P ≤ 0.05).

Results and discussion

Base levels of ech42 and nag1 expression in the non-amended controls were 20.2 mU and 24.8 mU per mycelial surface area, respectively. We found evidence of a variety of responses to the different mycotoxins regarding expression of ech42 and nag1 and Trichoderma growth (Table 2). Fumonisin B1 and patulin had no impact on expression of either chitinase gene or on growth. Ochratoxin A repressed nag1 expression by 17 % but had no effect on ech42. Aflatoxin B1 and α-zearalenol induced ech42 expression by 18 % but had no effect on nag1. Beauvericin had no effect on gene expression but significantly reduced Trichoderma growth. No mycotoxin had an effect on the phenotype (i.e., very compact growth) or on sporulation of Trichoderma.

Table 2. Summary of mycotoxin effects on growth and chitinase gene expression in T. atroviride P1.

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Trichoderma growth</th>
<th>Trichoderma chitinase gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ech42</td>
<td>nag1</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>100.0</td>
<td>117.4*</td>
</tr>
<tr>
<td>Beauvericin</td>
<td>89.7*</td>
<td>93.7</td>
</tr>
<tr>
<td>Fumonisin B1</td>
<td>103.0</td>
<td>97.5</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>102.6</td>
<td>96.9</td>
</tr>
<tr>
<td>Patulin</td>
<td>100.4</td>
<td>95.0</td>
</tr>
<tr>
<td>α-Zearalenol</td>
<td>99.1</td>
<td>119.8*</td>
</tr>
<tr>
<td>Deoxynivalenol a</td>
<td>100.0</td>
<td>100.8</td>
</tr>
</tbody>
</table>

T. atroviride P1 was grown in presence of different mycotoxins amended on malt extract agar plates. Growth and expression are expressed as % of the control. Asterisks (*) indicate means that are significantly different from the non-mycotoxin amended control according to t-tests (P ≤ 0.05). Results were based on three experiments with four replicates each.

For practical reasons, the concentration used were determined by duplicating the tolerable levels in food (according to Swiss regulations)(Table 1), assuming a certain environmental relevance of these concentrations. In immediate neighborhood of the producing fungi, mycotoxin concentrations are potentially much higher than the overall concentration measured in food. The use of these relatively low concentrations has some consequences for the interpretation of the results described. The extent, but not the manner of changes in gene expression mediated by substances is often dependent on the concentration used (Lutz et al. 2003; Notz et al. 2002). Thus, the effects observed in this study might are higher at the direct interaction court. On the other hand, some interactions may not have been detectable due to the relatively low mycotoxin concentrations used.
Secondary metabolites, such as e.g. mycotoxins, can contribute to the ecological fitness of pathogens by reducing growth of microbial competitors and/or by reducing the expression of inhibitory factors in the antagonists (Duffy et al. 2003). In this study, beauvericin reduced growth of the antagonist and ochratoxin A repressed expression of one chitinase gene. This indicates that these mycotoxins might contribute to the producer’s competitiveness. Furthermore, we found the first example of pathogen toxins that may also serve as positive signals up-regulating antagonist gene expression. In much the same way that breakdown products of fungal cell walls trigger chitinase gene expression (Zeilinger et al. 1999), aflatoxin B1 and α-zearalenol appear to function as chemical cues that might alert the mycoparasite to host presence.

Mycotoxin-mediated control of antagonist gene expression has potential consequences for the application of Trichoderma as a biocontrol agent. Antagonist response to these and other pathogen toxins provides a plausible explanation for some of the variability observed in performance across different environments. Our findings demonstrate the potential importance of including mycotoxin interactions at the gene expression level as a criterion in designing selection strategies for antagonist strains to biologically control mycotoxigenic fungi.

Acknowledgements

We are grateful to Matteo Lorito for generously providing T. atroviride strains, and Sheri Woo and Robert Mach for advice on the GOX measurements. This research was supported by the European Union COST Actions 830 and 835, the Swiss National Foundation for Scientific Research (Project 5002-57815) and the Swiss National Centre of Competence in Research (NCCR Plant Survival, Neuchâtel).

References


Biological control of soil-borne diseases in flowerbulb cultivation in the Netherlands

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Abstract: Flowerbulb production in the Netherlands is threatened by several soil-borne diseases. Root rot caused by Pythium spp., Augusta disease caused by Tobacco necrotic virus (TNV), and bare patch disease caused by Rhizoctonia solani are major problems. Biological control methods are being investigated at Applied Plant Research Flowerbulbs.

The biosurfactant producing Pseudomonas fluorescens strain SS101, was shown to cause the lysis of zoospores of oomycetes. In bioassays, SS101 suppressed Pythium root rot in hyacinthus, iris and crocus under controlled and under field conditions. In several expanded field trials with hyacinths, the efficacy of biocontrol varied by field and by year. Augusta disease in tulips is transmitted via zoospores of the fungal vector Olpidium brassicae. Adding strain SS101 to the standard fungicide solution, which is used for bulb drench prior to planting, resulted in a 46% reduction of the disease incidence compared to a bulb drench without the antagonist. The application of SS101 is currently repeated and further optimized in new experiments.

Verticillium biguttatum is an obligate mycoparasite of R. solani. The antagonist infects the sclerotia and the hyphae of its host. R. solani anastomosis group (AG) 2-2IIIB causes stem and bulb infection in lily, resulting in reduction of bulb yield and quality. In lab experiments, the antagonist completely suppressed the hyphal growth of R. solani AG2-2IIIB through soil at 24°C. In field trials, a spore suspension of V. biguttatum was applied as a bulb drench and a soil treatment prior to planting, resulting in significant control of Rhizoctonia with artificial inoculum as well as with natural infestation.

Key words: flowerbulbs, biological control, antagonists, Pythium spp., Augusta disease, Tobacco necrotic virus, Pseudomonas fluorescens, biosurfactant, Olpidium brassicae, Rhizoctonia solani, Verticillium biguttatum

Introduction

Several soil-borne diseases threaten the production of bulbs in the Netherlands. Measures to control these diseases are not always sufficient, are not available or require a high input of pesticides. Applied Plant Research investigates the development of integrated management strategies to control these diseases. Integrated management combines preventive and control measures. The contribution of biological control by Pseudomonas fluorescens in integrated management of the soil-borne diseases Pythium root rot, Rhizoctonia bare patch disease and Augusta disease was investigated. Furthermore, the role of the fungus Verticillium biguttatum to control Rhizoctonia bare patch disease is discussed.

Root rot caused by oomycetes of the genus Pythium is an important disease in flower bulb cultivation in the Netherlands. This root rot can be caused by several species of Pythium. There is no clear association between root rot of one bulb species, e.g. Hyacinthus, and a specific Pythium species. Pythium root rot can lead to substantial yield losses. Augusta disease, caused by Tobacco necrotic virus (TNV) and its vector Olpidium brassicae, is a severe disease in tulips. The virus is spread via zoospores of the fungus and this is also its
means of entrance into the roots. The spread and establishment of *O. brassicae* are favoured by a water rich environment. At present, there is no chemical control and disease management depends on cultural practices. Heavy soils and early planting of tulips in the season increase chances of infection. Also Augusta disease can lead to considerable yield losses and reduction of bulb quality. At present, it is recognized as the most important viral disease in tulips.

Lily is susceptible to *Rhizoctonia solani* Kühn, anastomosis group (AG) 2-2IIIB, which causes subsurface stem and bulb infection, resulting in premature die-off and reduction of both bulb yield and quality. At present, no effective fungicides are available for control of *Rhizoctonia* in lily and losses mount up to six million euro per year.

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Figure 1: Bulbweight (g/bulb) of *Hyacinthus* (cv. Pink Pearl) in field experiments conducted over three consecutive seasons, i.e. 2001-2002 (bars without pattern), 2002-2003 (bars with dotted pattern) and 2003-2004 (bars with striped pattern). *Pseudomonas fluorescens* strain SS101 was applied by pouring a bacterial suspension over the planted bulbs, resulting in a final density of $10^7$ CFU/g soil for 10-cm soil layer on top of which the bulbs were planted. The fungicide (Ridomil Gold with metalaxyl as the active ingredient) was applied at standard dosage (1.25 l/ha). The experiments were carried out in field soil that consisted of 10% naturally infested field soil. Each treatment was repeated four times. Differences in bulb weight between the treatments were evaluated for each season by the analysis of variance (ANOVA). T-tests were used to determine pair-wise differences ($P \leq 0.05$). For each season, different letters indicate statistically significant differences. All calculations were performed using the statistical programme Genstat 5 (Genstat 5 Committee, 1993; Goedhart *et al.*, 1992).

### Results and discussion

*Pseudomonas fluorescens*

Antagonistic *Pseudomonas* spp. strains were tested for their disease suppressive abilities against *Pythium* root rot. Under controlled conditions in greenhouse experiments, strains producing the antibiotic 2,4-diacetylphloroglucinol (Phl) or producing biosurfactants showed significant control of *Pythium* root rot of different bulb crops (*Iris, Crocus, Hyacinthus*).
Biosurfactant-producing strain SS101 almost completely prevented Pythium root rot in *Hyacinthus* and *Crocus* (Souza et al. 2003) A mutant of strain SS101, defective in biosurfactant production, provided substantially less control of Pythium root rot in *Hyacinthus*, suggesting that biosurfactant production plays an important role in disease control.

In bioassays under field conditions, both the biosurfactant-producing strain SS101 and a Phl-producing strain were able to control disease of different crops (*Hyacinthus*, *Iris* and *Crocus*). The disease suppression by biosurfactant producing strain SS101 was studied more intensively in several field trials. Also in these field trials, SS101 was able to control *Pythium* root rot of *Hyacinthus*. However, the efficacy varied from field to field and from year to year. Nevertheless, application of the biosurfactant producing strain resulted overall in a higher bulb yield as compared to the nontreated control, and was comparable to the effects obtained by standard fungicide application (Figure 1). In conclusion, since good control can be achieved by the application of this *Pseudomonas* strain under field conditions the method of application is currently being optimized.

![Figure 2. Percentage of healthy tulip plants at the end of the growing season after different treatments prior to planting. Tulip bulbs (cv. Angelique) were dipped for 15 minutes in a bacterial suspension (*P. fluorescens* SS101; 10^8 cfu/ml), in a standard cocktail of fungicides (0.5% Captan (546 g), 4% carbendazim (500 g), 0.3% prochloraz (450 g/l), 0.25% shirlan (250 ml/l), 0.2% ronilan (200ml/l)) or in a standard cocktail of fungicides supplemented with *P. fluorescens* SS101 (10^9 cfu/ml). Bulbs treated with water served as a control. For each treatment, bulbs were planted in naturally Augusta infested soil in plastic baskets (28 cm x 28 cm x 30 cm, l x b x h). During the growing season, crop stand was monitored and at the end of the season the percentage of healthy tulip plants (without TNV symptoms) was scored. Each treatment was repeated four times. The percentage of healthy plants was evaluated by analysis of variance (ANOVA) followed by a T-test. Different letters above the bars indicate a statistically significant difference (P<0.05).](image-url)

Since the biosurfactant-producing strain SS101 has zoosporicidal activity (Souza et al., 2003), the activity of this strain was also investigated against *Olpidium brassicae*. The zoospores of *O. brassicae* are essential for transfer of Tobacco necrosis virus (TNV), the...
causal agent of Augusta disease in tulips. In practice, tulip bulbs are dipped into a solution of fungicides prior to planting to prevent diseases. The effect of *P. fluorescens* SS101 was tested in a field experiment where tulip bulbs were dipped in different solutions prior to planting in naturally Augusta infested soil (Figure 2). Dipping of the tulip bulbs in suspensions of either SS101 alone or a cocktail of fungicides did not result a disease reduction relative to the control. However, when both treatments were combined the percentage of healthy tulips at the end of the growing season was strongly increased. *In vitro* studies further showed that growth of *P. fluorescens* strain SS101 was delayed when combined with fungicides but restored after 1-2 days of incubation.

*Verticillium biguttatum*

*Verticillium biguttatum* Gams is an obligate mycoparasite of various pathogenic anastomosis groups of *R. solani*. The antagonist has shown its potential as a biological control agent of black scurf caused by *R. solani* AG3 in potato (Jager et al., 1991) and stunt disease in barley, caused by *R. solani* AG8 (Morris et al., 1995). The antagonist requires specific conditions for obtaining reliable effects (Van den Boogert & Velvis 1992). Environmental factors may impede its activity, and failures of control have been attributed e.g. to low temperatures or high pH. *V. biguttatum* infects the sclerotia as well as hyphae of its host (Van den Boogert et al., 1989).

Figure 3. Effect of *Verticillium biguttatum* and the fungicide azoxystrobin on relative bulb yield (A) and percentage healthy bulbs (B) in a field experiment with lily in soil naturally infested with *Rhizoctonia solani* AG2-2IIIB. Three treatments with four replicates per treatment were placed in randomized blocks on sandy soil (4% organic matter, pH7): untreated control, a soil treatment with *V. biguttatum* (10^10 conidia/m^2) combined with a bulb drench (submerging the bulblets in a suspension of 10^10 conidia/l) and a fungicide treatment with azoxystrobin (Amistar 6 l/ha, 250 g/l a.i., Syngenta). Bulb yield and percentage infected bulbs were determined after harvest in November. For statistical analyses, data on bulb infection were converted to percentages and a binomial regression using a generalized linear model (GLM) and a logit link was performed in order to determine treatment effects. Bulb yields were evaluated by the analysis of variance (ANOVA). *T*-tests were used to determine all pair-wise differences of means at significant level *P*≤0.05. All calculations were performed using the statistical programming language Genstat 5 (Genstat 5 Committee, 1993; Goedhart *et al.*, 1992). For each panel, different letters indicate a statistically significant difference.
In this study, the potential of *V. biguttatum* to control *R. solani* AG2-2IIIB in lily was investigated. In a lab experiment, the antagonist completely suppressed the hyphal growth of *R. solani* AG2-2IIIB through soil at 24°C. In the field, two main inoculum sources can be distinguished for Rhizoctonia infection of lily: bulb-borne inoculum and soil-borne inoculum. Therefore, a spore suspension of *V. biguttatum* was applied as a bulb drench and as a soil treatment prior to planting. This combined treatment resulted in significant control of *R. solani* AG2-2IIIB in small scale experiments in sandy soil with artificial inoculum. In larger scale field experiments with naturally Rhizoctonia-infested soil, *V. biguttatum* also gave good control (Figure 3). When the percentage of healthy bulbs is considered, *V. biguttatum* performed even better than a standard application of the fungicide azoxystrobin (Figure 3B).

*V. biguttatum* had no effect on infection of *R. solani* AG2-t in tulip. Tulips are planted in autumn and susceptible to stem and leaf infection by AG2-t during winter and early spring when soil temperatures are substantially lower than the minimum temperature of 13°C for *V. biguttatum*.

Currently the different biological control agents discussed above are developed further for use in both organic and integrated management of soil-borne diseases. An obstacle for official authorisation of *V. biguttatum* or *P. fluorescens* SS101 as biological control agents may be the limited sales potential for a relatively small market. At the moment, *V. biguttatum* is one of the potential candidates for a project by which official registration of biological control agents should be facilitated. Large scale production, application and more fundamental research on genetics *P. fluorescens* SS101 is currently investigated by Jos Raaijmakers and colleagues.

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Regulation of beneficial traits in antagonistic bacteria

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Abstract: The phz operon of Pseudomonas fluorescens 2-79, which produces the antibiotic phenazine-1-carboxylic acid (PCA), is preceded by phzR and phzI, a pair of quorum-sensing members of the luxR-luxI gene family. Quantitative analyses showed that strain 2-79 produces six acyl-homoserine lactone (HSL) signaling components, of which N-(3-hydroxyhexanoyl)-L-HSL is the most abundant. Deletion of phzR and phzI led to the loss of production of PCA as well as all acyl-HSLs. Expression of phzI in Escherichia coli or the PCA nonproducer P. fluorescens 1855 enabled the synthesis of all six acyl-HSLs normally produced by strain 2-79. A reporter strain of 2-79 bearing phzA and phzR fused respectively to lacZ and uidA required PhzR and the addition of acyl-HSLs for maximum expression of both gene fusions. Analyses with synthetic acyl-HSLs revealed that the phzA::lacZ fusion responded with highest sensitivity and greatest magnitude to N-(3-hydroxy-hexanoyl)-L-HSL. When exposed to extracts of culture supernatants from strain 2-79 containing normal ratios of all six acyl-HSLs, the reporter responded to N-(3-hydroxy-hexanoyl)-L-HSL but not to the other five acyl-HSLs. Mapping of the transcriptional start sites for the divergently-oriented phzA and phzR genes showed that the putative -35 element of the phzA promoter is situated downstream from and adjacent to an 18-bp almost-perfect inverted repeat, the phz-box, which resembles the binding sites of other members of the LuxR family. Disrupting the phz-box abolished PhzR-dependent activation of phzA and phzR. We conclude that PhzI of strain 2-79 synthesizes 3-OH acyl-HSLs and, unlike the N-hexanoyl-L-HSL-based phz systems of P. aureofaciens 30-84 and P. chlororaphis PCL1391, strain 2-79 recognizes N-(3-hydroxy-hexanoyl)-L-HSL as its quorum-sensing signal. We conclude further that PhzR, together with its quormone, activates expression of phzA and phzR and that this activation requires the phz-box present in the divergent promoter region. Considering the differences in spacing of the phz-box relative to the -35 elements of the phzA and phzR promoters, we also suggest that PhzR activates transcription of these genes by different mechanisms.

Key words: phenazines, quorum sensing, acyl-homoserine lactone, gene expression, antibiotics

Introduction

Plant pathogens reduce the productivity of food, fiber, and ornamental crops worldwide. Plant disease prevention remains heavily dependent on the use of agricultural chemicals [Hall & Mann, 1999] even though many countries already have banned the use of certain pesticides and are scheduled to reject others from the market. Biological control is the best alternative for managing root diseases for which there are no resistant varieties and chemical treatments are limited. Prominent among the benefits of biological control is the reduced use of synthetic pesticides, many of which can persist and adversely affect non-target organisms in the environment [Cook, 1993].
Biological control of plant root pathogens often is based on the use of plant growth-promoting rhizobacteria (PGPR) [Kloepper & Beauchamp, 1992], beneficial free-living soil microorganisms that colonize roots and stimulate plant growth. Plant growth promotion can result in better growth, seed emergence, plant weight, crop yield, and disease control. Major mechanisms underlying disease control include induction of host plant defense mechanisms (ISR) and pathogen suppression through parasitism/predation, competition, or antibiosis. The latter is the inhibition or destruction of an organism by a metabolite produced by another organism, and results from the synthesis of antibiotics, low molecular-weight organic compounds of microbial origin that are deleterious, even at low concentrations, to the growth or metabolic activities of other microorganisms.

Among the most intensively studied antibiotic-producing biocontrol agents are strains of *Pseudomonas fluorescens* from soils naturally suppressive to *Gaeumannomyces graminis* var. *tritici*, which causes take-all disease of wheat [Weller et al., 2002]. These strains, and closely related isolates of *P. chlororaphis* and *P. aureofaciens*, produce biologically active metabolites that not only serve as major determinants in the control of soilborne plant pathogens, but also contribute to the ecological competence of the strains that produce them [Thomashow & Weller, 1995].

Despite increased interest in biological control, there are very few commercially available biocontrol products, and they have a low market share. This is due partially to variations in the performance of PGPR, particularly in the field. A better understanding of the genes required for the synthesis of antibiotics by PGPR, and the mechanisms by which their expression is regulated, is needed for the development of biocontrol agents that perform more consistently. Knowledge of the regulation of antibiotic production by PGPR, however incomplete, has grown immensely in recent years, and it is impossible to cover all aspects of the topic in this paper. Therefore, we will focus here on certain aspects in regulation of the synthesis of PCA by the model biocontrol agent *P. fluorescens* 2-79.

**Materials and methods**

**Bacterial strains and growth conditions**

All *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C or 28°C. Strains of *P. fluorescens* were grown in King’s medium B [King et al., 1954], ABM minimal medium [Shaw et al., 1997] or LB medium at 28°C. The bioreporter strains *Agrobacterium tumefaciens* NTL4(pZLR4) [Cha et al., 1998] and *Chromobacterium violaceum* CV026blu [McClean et al., 1997] were grown at 28°C in ABM and LB medium, respectively. Antibiotics were added to final concentrations, in µg ml⁻¹, of: carbenicillin, 100 or 200; streptomycin or spectinomycin, 100; kanamycin, 50; gentamicin, 15 or 50; rifampicin, 75; and tetracycline, 12.5 or 25.

**DNA and gene replacement manipulations**

Standard methods were used for plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation [Ausubel et al., 2002]. Mutants of *P. fluorescens* were constructed by a strategy [Schweizer, 1992] in which the gene of interest was cloned, interrupted with a tetracycline resistance gene, and recombined into the genome. The deletion replacement event was confirmed by PCR analysis. Transcription start sites were mapped by primer extension essentially as described by Ausubel et al. [Ausubel et al., 2002].

**Metabolite extraction and analysis**

Phenazine compounds were extracted from cultures of *P. fluorescens* and its mutants and analyzed by high performance liquid chromatography as described previously [Mavrodi et al., 1998; Mavrodi et al, 2001]. Acyl-HSLs were extracted from culture supernatants with
ethyl acetate or dichloromethane, dried under vacuum, dissolved in HPLC-grade ethyl acetate or dichloromethane, and analyzed by thin-layer chromatography (TLC) on C18 reversed-phase plates developed in methanol:water (60:40) as described previously [Cha et al., 1998]. After chromatography, the plates were air-dried, overlaid with appropriate indicator strains, and inspected visually after 15-18 hours at 28°C. The diameters of the spots from acyl-HSLs present in the mixture were measured and compared with spots from standard compounds of known concentrations separated on the same plate.

Results and discussion

**Production of phenazine antibiotics by P. fluorescens 2-79 requires PhzR and PhzI.**

A conserved seven-gene operon, \( phzABCDEFG \), encodes for the synthesis of PCA from chorismate in all known phenazine-producing fluorescent pseudomonads. In *P. fluorescens*, *P. aureofaciens*, and *P. chlororaphis*, the linked genes \( phzI \) and \( phzR \) perform acyl-HSL mediated regulation of \( phz \) gene expression [Mavrodi et al., 1998; Chin-A-Woeng et al., 2001; Wood & Pierson, 1996].

![Gene structure of the *phz* locus from *P. fluorescens* 2-79 and its mutants, 2-79IR, 2-79Z, 2-79R, and 2-79IZ (A). The structure and orientation of *phz* genes are shown below a restriction map of the locus. Arrows depict sites of insertion of tetracycline resistance cassette within *phzI* and *phzR* genes, and *phzD::lacZ* fusion. Production of phenazine-1-carboxylic acid by the wild-type 2-79 and its complemented regulatory mutants (B).](image)

*P. fluorescens* 2-79 produces at least six acyl-HSLs including the 3-hydroxy forms, N-(3-hydroxy-hexanoyl)-L-homoserine lactone (3-OH-C6-HSL), N-(3-hydroxy-octanoyl)-L-homoserine lactone (3-OH-C8-HSL), and N-(3-hydroxy-decanoyl)-L-homoserine lactone (3-OH-C10-HSL), the alkanoyl-forms hexanoyl-homoserine lactone (C6-HSL) and octanoyl-homoserine lactone (C8-HSL), and a rare seven-carbon acyl-HSL N-(3-hydroxy-heptanoyl)-L-HSL [Shaw et al., 1997]. Both *P. aureofaciens* 30-84 and *P. chlororaphis* PCL1391 have been reported to produce and utilize C6-HSL [Chin-A-Woeng et al., 2001; Wood et al., 1997]
but not 3-hydroxy acyl-HSLs. Because the amino acid sequences of the PhzI and PhzR proteins from the three strains are very similar, it became important to determine what acyl-HSL signals are produced by PhzI of *P. fluorescens* 2-79, and which of these activate the 2-79 PhzR transcription factor.

To determine if *phzI* and *phzR* are required for expression of the *phz* operon in strain 2-79, we constructed the mutant 2-79IR, in which *phzI* and *phzR* were replaced by a tetracycline resistance cassette (Fig. 1). Whereas the parent strain produced PCA, the deletion mutant failed to do so. Phenazine production was restored in 2-79IR upon introduction of a plasmid expressing cloned *phzI* and *phzR* genes, or only *phzR* in the presence of an ethyl acetate extract of the supernatant from a culture of wild-type strain 2-79 (Fig. 1). Combined, the data suggest that PCA production by *P. fluorescens* 2-79 is regulated by PhzR, and that this LuxR homolog requires a soluble autoinducer likely produced by PhzI.

**The *phzI* gene of strain 2-79 codes for the synthesis of both 3-hydroxy- and alkanoyl-acyl homoserine lactones**

Because strain 2-79 produces a complex array of acyl-HSL signals, we quantified the amounts of the major forms present in extracts of culture supernatants. In one such sample from a culture grown in minimal medium to late exponential phase, 3-OH-C6-HSL at a concentration of 1.3 µM dominated the mixture, while the alkanoyl signal C6-HSL was present at 0.075 µM and 3-OH-C8-HSL, at 0.04 µM, was the least abundant of the species tested. The absolute amounts of each species varied somewhat as a function of culture phase and medium, but their relative amounts remained the same (data not shown).

![Figure 2](image-url)

*Figure 2. The *phzI* gene of *P. fluorescens* 2-79 confers production of all six acyl-HSLs. Ethyl acetate extracts of culture supernatants of strains harboring the cloned *phzI* gene in pSF106 or the vector pDLB4 were separated and the TLC plates were overlayed with the *A. tumefaciens* (A) or *C. violaceum* (B). The lanes contain extracts from cultures of: (1) *P. fluorescens* 2-79; (2) *E. coli* DH5α(pDLB4); (3) DH5α(pSF106); (4) *P. fluorescens* 1855(pDLB4); (5) *P. fluorescens* 1855(pSF106). Lane 6 contains a sample of pure N-hexanoyl-HSL standard. The locations and identities of the six acyl-HSLs are indicated at the margins of each plate.*

To assess the role of *phzI* in the synthesis of these signals, culture supernatants of 2-79IZ, which lacks *phzI*, were analyzed (data not shown). No active signal compounds were detected by either the *A. tumefaciens* or *C. violaceum* reporter strains (Figs. 3A and B, lanes 3). In contrast, *E. coli* DH5α and *P. fluorescens* 1855, neither of which produce their own acyl-HSLs, synthesized all six 2-79 3-hydroxy- and alkanoyl-acyl-HSLs upon expression of the cloned *phzI* gene (Figs. 2A and B). Thus, we conclude that the PhzI enzyme catalyzes the synthesis of all six of the HSL derivatives produced by strain 2-79 (Fig. 2).
therefore differs from *P. aureofaciens* 30-84, which produces additional signals via a second acyl-HSL synthase, CsaI [Zhang & Pierson, 2001]. We were unable to detect a *csaI* homolog in the genome of strain 2-79 by low stringency hybridization with a *csaI* probe derived from strain 30-84 (data not shown). These results suggest that *phzR* and *phzI* comprise the only acyl-HSL-dependent regulatory system present in *P. fluorescens* 2-79.

**PhzR is maximally sensitive to 3-OH-C6-HSL**

To assess the response of PhzR to the various acyl-HSL signals produced by strain 2-79, we constructed a tandem reporter system in *P. fluorescens* 1855 comprised of *phzR* on pSF105 and the *phzR-phzA* divergent dual promoter region fused between oppositely-oriented *uidA* and *lacZ* reporter genes on pSF107. This reporter strain responded only to 3-OH-C6-HSL of the six acyl-HSLs present in extracts of culture supernatants from strain 2-79. The reporter might have detected one or more of the other five acyl-HSLs if we had chromatographed a more concentrated sample, but the results show clearly that PhzR can be activated by 3-OH-C6-HSL when the other acyl-HSLs present at physiological ratios do not elicit a detectable response. The *phzA::lacZ* reporter also responded to pure 3-OH-C6-HSL at concentrations more than 10-fold lower than those of C6-HSL and 3-OH-C8-HSL standards required to activate the reporter to a similar level. At saturating signal levels, cells grown with 3-OH-C6-HSL activated the reporter to levels almost twice those observed in cells grown with pure forms of the other acyl-HSLs.

**The phz-box is required for PhzR-mediated activation of phzA and phzR**

Consistent with a model in which PhzR regulates expression of the *phz* operon, the intergenic region upstream of *phzA* contains an 18-bp almost-perfect inverted repeat similar to the lux box of *V. fischeri* (Fig. 3). Such boxes act as binding sites for LuxR-type activators [Fuqua et al., 2001]. The sequence is identical to inverted repeats located upstream of the *phz* operon of *P. aureofaciens* 30-84 and *phzI* in 30-84 [Wood & Pierson, 1996] and *P. chlororaphis* PCL1391 [Chin-A-Woeng et al., 1998] (Fig. 3), although there is no evidence that these elements regulate gene expression in either strain. The location of *phz-box* also suggests that cis-active sequences upstream of the *phz* operon constitute a Class II promoter upon which PhzR acts as an ambidextrous activator, as proposed for LuxR, TraR, and LasR [Fuqua et al., 2001].

We evaluated the role of this site in transcription by introducing a 4-bp duplication of the central GATC of the putative *phz-box* present in the reporter plasmid pSF107. Expression of the *phzA::lacZ* fusion remained at basal levels in the mutated reporter strain, even when it was grown with 3-OH-C6-HSL. Similarly, the *phzR::uidA* fusion was induced almost two-fold in response to 3-OH-C6-HSL in a strain harboring the wild-type promoter in pSF107, but expression remained at basal levels in the strain harboring the mutant *phz-box* sequence in pSF108. Such relatively weak autoregulation is common among members of the *luxR* gene family [Piper et al., 1999] including *phzR* of strain 30-84, which activates expression of its own gene some 3.5-fold [Pierson et al., 1994]. The organization within the promoter region in strain 2-79 suggests that PhzR activates expression of *phzR* by a mechanism different from that used to activate the *phz* operon.

PhzR from *P. fluorescens* 2-79 is 88% and 87% identical to the PhzR proteins of *P. aureofaciens* 30-84 and *P. chlororaphis* PCL1391 respectively, whereas the PhzR proteins of strains 30-84 and PCL1391 are 93% identical. Sequence differences between PhzR from 2-79 and the other two pseudomonads are distributed over the entire lengths of the proteins. However, several variant residues are located in the N-terminal region, which in TraR and LuxR is required for acyl-HSL binding and ligand specificity [Cha & Winans, 2004; Luo et al., 2003]. One or more of these N-terminal substitutions may be responsible for the
difference in signal specificity exhibited by PhzR from strain 2-79. Similarly, the PhzI protein from strain 2-79 is 87% and 86% identical to the PhzI proteins from strains 30-84 and PCL1391 respectively, which themselves are 96% identical.

In summary, the expression of phz operon in *P. fluorescens* 2-79 is controlled by an autoinducer-type regulatory system composed of the LuxR homolog PhzR, and diffusible acyl-HSL signals produced by the LuxI homolog, PhzI. This regulatory ensemble resembles the systems that control phenazine gene expression in *P. aureofaciens* 30-84 [Wood & Pierson, 1996; Wood et al., 1997] and *P. chlororaphis* PCL1391 [Chin-A-Woeng et al., 2001]. However, whereas the latter two strains produce and respond to the alkanoyl signal C6-HSL [Wood et al., 1997; Chin-A-Woeng et al., 2001], *P. fluorescens* 2-79 produces at least six detectable acyl-HSLs, four of which are 3-OH derivatives. Moreover, the dominant signal produced by strain 2-79 is 3-OH-C6-HSL, not the alkanoyl-C6 species. While the core phz gene set and the phzR/phzI regulatory system are strongly conserved in these three rhizosphere-dwelling fluorescent pseudomonads, it is not clear is why the regulatory system of strain 2-79 has evolved a signal specificity differing from that of the other two strains.
pseudomonads. The additional acyl-HSLs synthesized by PhzI from 2-79 accumulate to levels much lower than the cognate signal 3-OH-C6-HSL, and they do not significantly affect activation of the phz operon even when provided at concentrations equal to that of the 3-OH-C6 signal. We suspect that these additional acyl-HSLs are products of relaxed substrate specificity of PhzI, a characteristic common among the members of the LuxI family of acyl-HSL synthases [Hwang et al., 1994; Pearson et al., 1994; Schaefer et al., 1996]. It is unlikely that these alternative signals have a significant role in modulating the expression of the phz operon in strain 2-79, but nonetheless they may influence acyl-HSL-dependent regulatory systems in other bacteria occupying the same habitat [Wood et al., 1997].

References

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Antagonistic activity among 2,4-diacetylphloroglucinol (DAPG)-producing fluorescent *Pseudomonas* spp.

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Abstract: Certain strains of *Pseudomonas fluorescens* that produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) can colonize roots and suppress soilborne pathogens more effectively than other strains that otherwise are almost identical. We recovered DNA fragments from the superior root colonizer *P. fluorescens* Q8r1-96 by suppression subtractive hybridization with the moderate colonizer strain Q2-87 in order to identify genetic differences that might account for superior colonization activity. One clone from Q8r1-96 exhibited similarity to a pore-forming bacteriocin and resides in a 23-kb pyocin-like gene cluster that includes a functional two-gene lysis module and a bacteriophage tail assembly. Treatment of strain Q8r1-96 with mitomycin C caused production of phage tail-like particles. Subsequent screening in vitro revealed that bacteriocin activity was common among 2,4-DAPG-producing isolates representing 17 distinct genotypes. Such activity may contribute to strain competitiveness and persistence in vitro and in the rhizosphere.

Key words: bacteriocin, rhizosphere, competitiveness, biological control

Introduction

Many strains of *Pseudomonas fluorescens* that produce 2,4-diacetylphloroglucinol (2,4-DAPG) are active as introduced biological control agents, and some have a major role in the natural suppressiveness of certain soils to fungal root pathogens [Raaijmakers et al., 2001; Weller et al., 2002]. Despite their overall similarity, these strains can be distinguished by genetic fingerprinting [McSpadden et al., 2000], and they also differ in their ability to colonize and persist in the rhizosphere of crop plants [Landa et al., 2003]. The molecular basis for these differences is of great interest because effective root colonization is an underlying prerequisite for biocontrol regardless of the mechanisms active in disease suppression. We have used genomic subtractive hybridization to detect differences between the genomes of two 2,4-DAPG-producing strains, Q8r1-96 and Q2-87, that differ in their ability to colonize the rhizosphere of wheat [Mavrodi et al., 2002]. Among the DNA fragments unique to the superior colonizer strain Q8r1-96 was one with DNA sequence similarity to colicin M, a bacteriocin from *Escherichia coli*. Bacteriocins, which are proteinaceous compounds produced by certain strains and that kill closely related strains or species [Riley et al., 2002], are the most abundant class of bacterial defensive factors. Many plant-associated *Pseudomonas* spp. can produce bacteriocins, and it has been suggested that such strains may have a competitive advantage in the rhizosphere [Parret et al., 2002]. We therefore questioned whether bacteriocin-mediated antagonism occurs among 2,4-DAPG-producing strains or contributes to their rhizosphere fitness.
Material and methods

Bacterial strains, plasmids, culture conditions, and genetic manipulations

The strains used in the study were described previously [Landa et al., 2002; McSpadden et al., 2000]. Cultures were grown in Luria-Bertani (LB) medium, tryptic soy broth (TSB) (Difco Laboratories, USA), or one-third strength King’s medium B (KMB). Standard techniques were used for plasmid DNA isolation, agarose gel electrophoresis, and transformation. For studies in soil, spontaneous rifampicin-resistant strains of P. fluorescens [Landa et al., 2002] were differentially tagged by electroporation with gentamycin and kanamycin resistance markers [Koch et al., 2001].

Antagonism in vitro

To test for bacteriocin production, each Pseudomonas strain was spread across a TSB agar plate, grown for 18 h at 28°C, and irradiated at 254 nm to induce bacteriocin production. The plates were incubated again for 3.5 h, growth was removed with a chloroform-soaked swab, and the plates were exposed to chloroform to kill the remaining cells. Indicator strains were then cross-streaked over the tester zone. Alternatively, tester cultures grown overnight at 28°C in LB broth were induced in fresh medium amended with 1 µg ml⁻¹ of mitomycin C, shaken for 5 hr, lysed with chloroform, spotted on LB agar, and overlaid with soft agar containing an indicator strain. Assay plates were incubated at 28°C and scored for inhibition at 12 and 24 h. Strains were tested twice and the combined results were analyzed with MVSP 3.12 software [Kovach Computing Services, UK] using a simple matching coefficient that considers both the presence and the absence of antagonism.

Rhizosphere colonization and competition

Wheat (Triticum aestivum L.) cv. Penawawa was sown in non-sterile soil as described previously [Landa et al., 2003]. Bacteria suspended in 1% methylcellulose were introduced to provide an initial density of ca. 1 x 10⁶ CFU g⁻¹ of soil or, for mixed inoculations, 0.5 x 10⁴ CFU g⁻¹ for each strain. Plants were incubated in a growth chamber for three successive 3-week cycles at 15°C with a 12-h photoperiod. Introduced strains on the roots of six plants were enumerated after each cycle by a modified dilution endpoint assay [McSpadden et al., 2000] in which bacteria in mixed inoculation treatments were selected in kanamycin- or gentamycin-amended media to distinguish between strains [Validov et al., 2005].

Results and discussion

Antagonistic activity is widely distributed among 2,4-DAPG-producers. Of 47 strains, only 11 did not antagonize another strain, and strains with a common genetic fingerprint (i.e., genotype) generally exhibited a similar breadth of activity. Strains of the P, D, E and I genotypes typically were broadly antagonistic, whereas members of the B and H genotypes failed to antagonize other strains. Isolates of the B, D, E, and P-genotypes generally were more susceptible to inhibition. Bacteriocins produced by Pseudomonas spp. include both low-molecular weight forms with enzyme activity and high-molecular weight particles resembling bacteriophage tails. To distinguish between these forms, lysates from DAPG-producing isolates were subjected to trypsin digestion, freeze-thaw, and ultrafiltration. Lysates from most of the antagonistic strains contained bacteriophage tail-like particles similar to R-pyocin bacteriocins from P. aeruginosa.

Because bacteriocins may help a strain to invade new habitats or repel other strains from already-occupied habitats [Riley et al., 2002], we examined the effect of antagonism on the competitiveness of 2,4-DAPG producers in the rhizosphere of wheat, a habitat to which they should be well-adapted. Studies included single and paired inoculations of five strains:
Pf-5 and Q2-87 (non-antagonistic in vitro); Q8r1-96 (antagonistic to Q2-87 and FTAD1R34); and FTAD1R36 (antagonistic to Q8r1-96 and Pf-5).

Population densities of single strains or strain pairs did not differ significantly immediately after inoculation. After one cycle, however, antagonists displaced the sensitive partners in all pairwise combinations except for Q8r1-96 + FTAD1R36. Population densities of the antagonists in these mixtures generally were not affected by the presence of the sensitive strains, whereas populations of the sensitive strains rapidly declined to below a detection limit of approximately log 3.26 CFU g\(^{-1}\) of root. These shifts are consistent with the inhibition of sensitive strains by their antagonists in the rhizosphere.

In paired inoculations, Q8r1-96 was largely unaffected by the presence of a competitor, and it displaced other strains regardless of whether they were sensitive (Q2-87 and FTAD1R34) nonantagonistic (Pf-5) or antagonistic (FTAD1R36) to it. Earlier rhizosphere competition studies [Landa et al., 2003] also showed that Q8r1-96 outcompeted MVP1-4r and 1M1-96 but was itself displaced from the rhizosphere by another 2,4-DAPG-producer, \(P. \textit{fluorescens}\) F113. All of the latter strains are neutral to one another in vitro.

Collectively, the results of this study suggest that bacteriocin production may contribute to competition between closely related strains of \(\textit{Pseudomonas}\) spp. in the rhizosphere, but it clearly is not the only factor involved. We speculate that Q8r1-96 may colonize roots more aggressively than other strains not only because of its antagonistic activity, but also because it more rapidly establishes a minimum threshold population required to overcome even bacteriocin-producing competitors to which it is sensitive.

A complete description of this study was recently published [Validov et al., 2005].

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References


Use of *Serratia plymuthica* to control fungal pathogens in bean and tomato by induced resistance and direct antagonism

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Abstract: We investigated the potential of *S. plymuthica* strain IC1270 to control the fungal leaf pathogens *Colletotrichum lindemuthianum* and *Botrytis cinerea* by induced resistance and direct antagonism. Bean and tomato plants grown in soil treated with IC1270 cells showed an enhanced resistance to the above pathogens. Mutant IC1270-P1 deficient in Prn production was as effective as the parental strain, indicating that there is no evidence for a role of Prn in induced resistance to *B. cinerea* in bean. However, IC1270-C7 mutant deficient in chitinase production was significantly less effective than the parental strain, suggesting the possible involvement of chitinolytic activity in induced resistance in bean plants. Leaf application with strain IC1270 decreased the number of *B. cinerea* spreading lesions from 92% in the control to 64%, and from 78% to 48% in bean and tomato, respectively, indicating that IC1270 is also a direct antagonist of this fungus. In addition, studies on *Serratia* metabolites that are involved in direct antagonism showed that pyrrolnitrin and chitinases are required.

Key words *Serratia plymuthica*; *Botrytis cinerea*; *Colletotrichum lindemuthianum*; Induced systemic resistance.

Introduction

Rhizosphere strain IC1270 of *Serratia plymuthica* produces a set of secreted chitinolytic enzymes, the antibiotic pyrrolnitrin, siderophore(s) and proteases (Ovadis et al. 2004). The bacterium was shown an effective biocontrol agent against several soil-borne and post-harvest fungal pathogens *in vitro* and under greenhouse conditions (Ovadis et al., 2004). In this work we investigated if the biocontrol potential of strain IC1270 includes also the ability to induce systemic resistance in plants.

Materials and methods

Pathogens

Inoculum of *Colletotrichum lindemuthianum* isolate 06/038 (race 385) was prepared as described by Bigirimana and Höfte (2001). *Botrytis cinerea* isolate R16, was maintained on Potato Dextrose Agar medium (PDA) at 24 °C under UV light with a photoperiod of 12h. The conidial suspension was diluted in a solution of 0.01 M glucose and 6.7 mM KH2PO4 to a final concentration of 10^5 conidia ml⁻¹ (Audenaert et al., 2002a; De Meyer and Höfte, 1997).
Plant material and experimental set-up
Bean or tomato seeds treated with bacteria or water were sown in a potting compost soil. The soil was mixed with bacterial inoculum to a concentration of $1 \times 10^8$ cfu g$^{-1}$. In the control treatment soil was treated with sterile demineralised water (Bigirimana and Höfte, 2002; Audenaert et al., 2002b).

Bioassay for foliar application
Bean plants were grown in untreated soil for 12 days before challenge inoculation with pathogen. *S. plymuthica* strain IC1270 or its mutants were cultured overnight on medium. Bacteria were collected and resuspended in physiological solution. Primary bean leaves were sprayed with bacterial suspension. Different IC1270 concentrations were further tested. Control leaves were treated with demineralised water. One day later, treated leaves were inoculated with *B. cinerea* suspension. Disease development was evaluated 5 days later by recording numbers of spreading and non-spreading lesions. Data were statistically analysed by logistic regression.

Results and discussion

Direct antagonism on bean leaves
Spraying bean leaves with strain IC1270 at $1 \times 10^6$ and $1 \times 10^8$ cfu ml$^{-1}$ significantly decreased the number of *B. cinerea* spreading lesions from 92% in the control to 64% and 77% respectively. Application of IC1270 at $1 \times 10^4$ cfu ml$^{-1}$ had no effect on disease incidence caused by *B. cinerea* (Fig. 1).

![Figure 1. Effect of foliar application of *S. plymuthica* IC1270 on susceptibility of bean to *B. cinerea*. C1 = $10^4$ cfu ml$^{-1}$; C2 = $10^6$ cfu ml$^{-1}$ C3 = $10^8$ cfu ml$^{-1}$. Bars with a common letter do not differ significantly using logistic regression test at P = 0.05.](image)

Metabolites involved in direct antagonism on bean
In bean, leaf application with strain IC1270 at $1 \times 10^6$ cfu ml$^{-1}$ significantly decreased the number of *B. cinerea* spreading lesions from 82.4% in the control to 45.7%, while all its mutants had no significant effect on disease incidence caused by *B. cinerea*. These results indicate that IC1270 is a direct antagonist of this fungus, and that pyrroloquinine and chitinases are required for antagonism (Fig. 2).

Direct antagonism on tomato leaves
In tomato plants, $1 \times 10^8$ cfu ml$^{-1}$ of IC1270 efficiently reduced *B. cinerea* spreading lesions from 78% to 48% (Fig. 3), while lower concentrations were not effective.
Figure 2. Effect of foliar application of wild type *S. plymuthica* IC1270, mutant P1 defective in pyrrolnitrin production, and mutant C7 defective in chitinase production, on the susceptibility of bean to *B. cinerea*. Bars with a common letter do not differ significantly using logistic regression test at $P = 0.05$.

Figure 3. Effect of foliar application of *S. plymuthica* IC1270 on the susceptibility of tomato to *B. cinerea*. C1 = $10^4$ cfu ml$^{-1}$; C2 = $10^6$ cfu ml$^{-1}$ C3 = $10^8$ cfu ml$^{-1}$. Bars with different letter are significantly different using logistic regression test at $P = 0.05$.

**Induced systemic resistance in bean towards *B. cinerea***

Strain IC1270 added in a concentration of $10^6$ cfu g$^{-1}$ soil was able to reduce the number of spreading *B. cinerea* lesions by 25% in comparison to the control plants (Fig. 4). The effectiveness of the strain was comparable to that of *Pseudomonas aeruginosa* KMPCH, a strain known to induce resistance in bean and tomato by the production of salicylic acid (De Meyer and Höfte, 1997; Audenaert et al., 2002b).

**Metabolites involved in induced systemic resistance**

To study metabolites involved in induced systemic resistance, specific knock-out mutants of IC1270 were tested for their ability to induced resistance relative to the parental strain, results showed that mutant IC1270-P1 deficient in Prn production was as effective as the parental strain in inducing resistance, while IC1270-C7 lacking chitinases had no significant effect on the *B. cinerea* infection (Fig. 5), indicating the involvement of chitinases in inducing resistance.
Figure 4. Effect of soil and seed treatment with *S. plymuthica* IC1270 and *P. aeruginosa* KMPCH on the susceptibility of bean to *B. cinerea*. Bars with different letter are significantly different using logistic regression test at $P = 0.05$.

Figure 5. Effect of soil and seed treatment with wild type *S. plymuthica* strain IC1270, mutant IC1270-P1 defective in pyrrolnitrin production, and IC1270-C7 defective in chitinase production on the susceptibility of bean to *B. cinerea*. Bars with different letter are significantly different using logistic regression test at $P = 0.05$.

Figure 6. Effect of soil treatment with *S. plymuthica* IC1270 and *P. aeruginosa* KMPCH on the susceptibility of bean to *Colletotrichum lindemuthianum*. Bars with different letter are significantly different using Mann-Whiney comparison test at $P = 0.05$.

**Induced systemic resistance on bean against *C. lindemuthianum***

The ability of IC1270 to induce resistance to *C. lindemuthianum* was compared to that of *P. aeruginosa* KMPCH, used as a positive control. IC1270 was as effective as KMPCH in inducing resistance to disease development caused by *C. lindemuthianum* (Fig. 6)

**References**


Role of *ptsP* and *orfT* genes in root colonization by *Pseudomonas fluorescens* Q8r1-96

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**Abstract:** *Pseudomonas fluorescens* Q8r1-96 produces 2,4-diacetylphloroglucinol (2,4-DAPG) and suppresses take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. Strain Q8r1-96 is representative of the D-genotype of DAPG producers, which aggressively colonize and maintain large population sizes on the roots of wheat, pea, and sugar beet. In order to study the role of *ptsP* and *orfT* in colonization of the wheat rhizosphere, clones containing these genes were identified in a Q8r1-96 genomic library, sequenced, and used to construct gene replacement mutants of Q8r1-96. PtsP plays a role in organic nitrogen utilization and the role of the *orfT* gene is unknown. Mutants in these genes were characterized phenotypically for 2,4-DAPG production, motility, fluorescence, colony morphology, exoprotease and hydrogen cyanide production, carbon and nitrogen utilization, and the ability to colonize the rhizosphere of wheat grown in a natural soil. Colonies formed by the mutant *orfT* did not differ from those of Q8r1-96, whereas those of the *ptsP* mutant exhibited altered colony morphology, increased fluorescence, and decreased motility. In addition, the *ptsP* mutant produced decreased amounts of exoprotease and phloroglucinol compounds as compared to the wild type. The *ptsP* mutant was impaired in wheat root colonization, whereas the *orfT* mutant was not impaired. However, both mutants were less competitive than *P. fluorescens* Q8r1-96 in the wheat rhizosphere when introduced into the soil in mixed inoculation with the parental strain.

**Key words:** rhizosphere colonization, *Pseudomonas* spp., *ptsP* and *orfT* genes

**Introduction**

Our studies of root colonization in the wheat rhizosphere have focused on *Pseudomonas fluorescens* Q8r1-96, which produces the polyketide antibiotic 2,4-DAPG and belongs to an important group of PGPR that suppresses root and seedling diseases on a variety of crops and plays a key role in the natural biological control of take-all disease of wheat known as take-all decline (Raaijmakers et al., 1997). Strain Q8r1-96 is representative of the D genotype of 2,4-DAPG producers as defined by restriction fragment length polymorphism (RFLP) analysis of the *phlD* gene (*phlD*+) (Mavrodi O. V. et al., 2001) and rep-PCR using the BOXA1R primer (BOX-PCR) (McSpadden Gardener et al., 2000). Strain Q8r1-96 is able to establish and maintain large population sizes (up to $10^7$ CFU/g of root) on the roots of wheat, pea (Raaijmaker and Weller, 2001), and sugar beet (Bergsma-Vlami, 2005) even when introduced at low doses.

Root colonization by introduced PGPR is a complex process that includes interactions among the introduced strain, the pathogen, and the indigenous rhizosphere microflora. Intensive research has indicated that colonization traits such as flagella, fimbriae, and the synthesis of the O-antigen of lipopolysaccharide, are involved in the attachment of *Pseudomonas* cells to plant roots. Genes responsible for the biosynthesis of amino acids, vitamin B1, a putrescine transport system, the NADH dehydrogenase NDH-1, the ColR/ColS two-component regulatory system, and a site-specific *sss* recombinase can influence the efficiency with PGPR colonize roots (Lugtenberg et al., 2001).
Recent findings that some bacterial genes are determinants of pathogenicity in such
different hosts as plants and animals (Rahme et al., 2000) have revealed unexpected
commonalities in the ways that bacteria interact with their hosts, and even more fundamental
similarities have become apparent with evidence of type III secretion genes in the PGPR
strain SBW25 (Rainey and Preston, 2000) and related genes in many other PGPR. This led us
to hypothesize that genes broadly implicated in interactions between bacterial pathogens and
their hosts might contribute to the exceptional ability of strains such as Q8r1-96 to colonize
and persist in the rhizosphere. A goal of this work was to assess the role of orfT and ptsP in
root colonization by Q8r1-96. These genes contribute to the pathogenicity of Pseudomonas
aeruginosa in both plant and animal systems (Rahme et al., 2000) and are highly conserved
(identity more than 70%) in the genomes of saprophytic rhizosphere pseudomonads.

In this study we identified and characterized the orfT and ptsP orthologues in P.
fluorescens Q8r1-96. Mutants in each gene were generated and used to evaluate the role of
the genes in colonization and strain competitiveness on the roots of wheat grown in
nonpasteurized soil, in the presence of indigenous rhizosphere microflora.

Material and methods

Bacterial strains, plasmids, and growth conditions
A spontaneous rifampicin-resistant derivative of Q8r1-96 was used for gene replacement
mutagenesis, while Q8r1-96Gm, a rifampicin-resistant derivative tagged with mini-Tn7-gfp2
was used in all competitive colonization experiments. Escherichia coli TOP 10 was used for
cloning experiments. P. fluorescens and E. coli strains were grown at 28°C and 37°C,
respectively, in Luria-Bertani (LB) medium, Pseudomonas agar F (PsF), Pseudomonas agar P
(PsP), or King’s medium B (KMB). Antibiotic supplements were used at the following
concentrations: ampicillin, 100 or 40 µg ml⁻¹; rifampicin, 100 or 90 µg ml⁻¹; tetracycline, 10 or
12.5 µg ml⁻¹; gentamycin, 2 µg ml⁻¹; cycloheximide, 100 µg ml⁻¹, chloramphenicol, 13 or 35 µg
ml⁻¹; and kanamycin, 25 or 50 µg ml⁻¹.

The genomic library of Q8r1-96 was constructed with partially Sau3AI-digested genomic
DNA cloned into the broad host-range cosmide vector pCPP47. The orfT and ptsP mutants were
generated by allelic replacement using pNOT19 and pMOB3 vectors. Mutants were confirmed
by PCR with different sets of primers.

DNA manipulations
Standard methods were used for plasmid DNA isolation, restriction enzyme digestion, agarose
gel electrophoresis, ligation, and transformation. PCR amplifications were carried out with Taq
or KOD Hot Start DNA polymerases according to the manufacturers’ recommendations. Total
DNA of P. fluorescens Q8r1-96 was isolated by using the Marmur procedure.

Production of additional extracellular metabolites
Siderophore production was determined by measuring orange halos after 2 days of growth at
28°C on CAS plates. Exoprotease production was detected by spotting 5 µl of an exponentially
growing bacterial culture adjusted to an OD₆₀₀ of 0.1 on skim milk agar. A clearing zone
surrounding the bacterial growth that is indicative of protease production was measured after 48
and 72 hrs of incubation at 28°C.

Motility assays
Motility assays were performed on LB medium solidified with 0.3% agar. The diameter of
outward expansion was measured 24, 48, and 72 h after inoculation.

Rhizosphere colonization assays
Rhizosphere colonization assays were performed in Quincy virgin soil. Bacterial inocula were
prepared in a 1% methylcellulose suspension to give 1 x 10⁴ CFU g⁻¹ of soil when strains were
introduced alone and ~ 0.5 x 10^4 CFU g⁻¹ of soil in mixed inoculation treatments contained a 1:1 mixture of Q8r1-96Gm and mutant. The actual density of each strain was determined by assaying 0.5 g of inoculated soil as described by Landa et al. (2002a). Six pre-germinated seeds of spring wheat (Triticum aestivum L.) cv. Penawawa were sown in pots containing 200 g of Quincy virgin soil inoculated with one or both bacterial strains. Wheat was grown for six successive two-week cycles in a controlled-environment chamber at 15°C with a 12-h photoperiod. After two weeks of growth (one cycle), one randomly selected plant was harvested from each replicate pot and the whole root system was prepared to determine the population size of the introduced bacteria as described by Landa et al. (2002a).

Results and discussion

The P. fluorescens Q8r1-96 orfT and ptsP genes studied in this work were chosen for analysis based on their role in interactions between P. aeruginosa PA14 and Arabidopsis (Rahme et al., 2000). The orfT mutant did not differ from the wild type in its ability to colonize the rhizosphere of wheat when it was introduced into soil alone, but it colonized significantly less than the parental strain when the two strains were introduced together. Inactivation of orfT resulted in a phenotype with reduced motility. On the other hand, the orfT mutant did not differ from the wild type in the production of exoprotease, hydrogen cyanide, siderophores, and phloroglucinol-related compounds. Further, the mutant was indistinguishable from P. fluorescens Q8r1-96 on PsF and LB media supplemented with 2% glucose, but was slightly more mucoid on PsP medium (data not shown).

Computer analyses of OrfT indicated that its orthologues are present in all Pseudomonas genomes sequenced to date. The predicted proteins share a HRDxxxN motif with eukaryotic and some prokaryotic Ser/Thr and Tyr protein kinases and some aminoglycoside phosphotransferases that are responsible for bacterial resistance to aminoglycoside antibiotics such as streptomycin and kanamycin. Despite these findings, this eukaryote-like protein kinase motif is too generic to provide any clues to the specificity of OrfT, and similarity to aminoglycoside phosphotransferases is partial and limited to about 40% of the polypeptide chain. Thus, the exact function of orfT in the rhizosphere competence of P. fluorescens Q8r1-96 at this point remains unclear.

In contrast to the orfT mutant, the rhizosphere competence of Q8r1-96 ptsP was strongly impaired, and its rhizosphere population densities were significantly lower than those of the wild type not only in mixed but also in single inoculations. The ptsP mutant exhibited altered morphology, reduced motility and exoprotease production, and an increased level of fluorescence. It produced significantly lower amounts of monoacetylphloroglucinol and 2,4-diacytethylphloroglucinol (data not shown).

The product of ptsP forms part of an alternative phosphoenolpyruvate (PEP): carbohydrate phosphotransferase (PTS) system. PTSs are present in a wide range of Gram-positive and Gram-negative microorganisms and have been best studied in enteric bacteria, where they are involved in sensing, transport and metabolism of carbohydrates, as well as in catabolite repression and inducer exclusion. It was proposed that EII^Ntr, together with EI^Ntr and NP^r presumably forms an alternative PTS system in P. putida and functions as a global rather than a promoter-specific regulatory factor and does not directly participate in phosphorylation or the utilization of carbohydrates (Rabus et al., 1999).

In conclusion, the data presented in this study suggest novel functions for two genes, ptsP and orfT, that previously were described as pathogenesis-related in P. aeruginosa. The ptsP and orfT mutants of Q8r1-96 do not have nonspecific growth defects in vitro (data not shown), and the effect of mutations became apparent only when mutants were tested in the rhizosphere in competition with the parental strain. To our knowledge, this is the first report
to provide evidence for the involvement of *ptsP* in rhizosphere colonization by fluorescent pseudomonads. Based on observations made in other bacteria, we speculate that PtsP may be involved in the regulation of amino acid metabolism in *P. fluorescens* Q8r1-96. This could explain the crucial role of this gene in rhizosphere competence, because amino acids represent one of the major components of root exudates and are thought to be among major sources of nitrogen, carbon, and energy for rhizosphere microorganisms (Lugtenberg *et al.*, 2001).

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**References**


The potential for *Meloidogyne graminicola* biological control in rice under oxic and anoxic soil environments

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Abstract: The rice root-knot nematode (*Meloidogyne graminicola*) is a serious pest in rice producing areas of South and Southeast Asia. A biological control system for *M. graminicola* is being developed at the University of Bonn using bacteria recovered from rice roots grown in upland and lowland rice soils of Bangladesh, Myanmar, and Taiwan. One of these bacteria, *Bacillus megaterium*, was found to have high activity against *M. graminicola* in the rice root resulting in a greater than 40% reduction in nematode galling severity and J2 penetration compared with noninoculated controls. *B. megaterium* also reduced attraction of the nematode to rice. This paper will discuss modes of action through which *B. megaterium* impacts *M. graminicola* and strategies for how biocontrol can be combined with floodwater management practices in rice to control nematode damage.

Keywords: bacteria, endophytic biocontrol, nematode, rice.

Introduction

Studies on tripartite interactions between a soilborne plant pathogen, its microbial antagonist(s), and the host plant have been largely restricted to aerobic agricultural systems, while there is no knowledge about such interrelationships in the anoxic environment surrounding a rice root. Understanding this unique environment is essential for assessing the capacity to implement biological control of root pathogens in rice, the dominant staple food crop of the developing world. Although chronic root disease is an important factor contributing to yield stagnation across major rice growing areas of South and Southeast Asia, few if any viable soilborne disease management options exist for this crop. Plant parasitic nematodes are the most important group of rice root disease organisms because they are well adapted to rice growing environments, and possess the ability to infect, survive, and reinfect new healthy roots as soils fluctuate between oxic and anoxic states.

*M. graminicola* is one of the most damaging of these flood-adapted nematodes, causing significant yield losses in upland, lowland rainfed, and deepwater rice ecosystems of South and Southeast Asia (Arayarungsarit, 1987; Bridge and Page, 1982; Netscher and Eralan, 1993; Padgham et al., 2004; Soriano and Reversat, 2003). In upland rice systems, the absence of floodwater provides no protection against *M. graminicola* invasion. In transplanted lowland rainfed rice, the nematode initially penetrates the root during aerobic nursery seedbed growth, and subsequent nematode invasion can occur during periodic soil dry-down periods in the post-transplant phase. In deepwater rice, *M. graminicola* invades during the pre-flooded rice establishment phase, which under heavy infection rates prevents the plant from later elongating above the rising floodwater resulting in high crop loss through drowning out.

Despite the widespread occurrence of *M. graminicola* in major rice producing areas, only limited research has been done on its biological control, and this has primarily focused on reducing the viability of the nematode in soil through the application of *Pasteuria*...
penetrans (Ahmed and Gowen, 1991; Duponnois et al., 1997). No research has been conducted on controlling *M. graminicola* through seedbed treatments with egg pathogens nor with *in-planta* means. Moreover, the influence that soil water conditions have on the survival and efficacy of biocontrol antagonists or how soil flooding can be used to strengthen biocontrol remain unknown.

Antagonist-nematode-host interactions have been extensively studied in aerobic agriculture, leading to the development of biocontrol systems that interfere with the nematode’s ability to find, penetrate, and complete its lifecycle in the host. This paper will describe a project recently initiated at the University of Bonn to develop a biological control system for *M. graminicola* on rice. This paper will also discuss how oxic and anoxic soil environments could possibly enhance endophytic biocontrol in the rice root.

**Materials and Methods**

**Bacterial isolates**

Endophytic bacteria were isolated from rice seedling roots grown in soil freshly collected from upland and lowland rice fields in Myanmar, and from lowland rice fields in Bangladesh and Taiwan. Several weeks after seedling emergence, roots were washed and bacteria isolation procedures performed. Roots were surface sterilized in 1% NaOCl and imprinted on TSA for a sterility check. Following sterilization, roots were ground with a sterile mortar and pestle, and the resulting solution plated on 5% TSA. Morphologically distinct isolates were obtained through subsequent transfers to 100% TSA. Bacterial species identification was performed using a MIDI-FAME analysis described by (Sasser, 1990). Following identification, all isolates were stored at −18°C in a glycerol-TSB media.

**Screening tests**

Thirty-one bacterial isolates were screened for their ability to reduce root-knot galling symptoms caused by *M. graminicola*. Only bacteria isolated from the inside of rice roots and not recovered in the sterility check were used for testing against the nematode. We consider these as putative endophytes.

One day-old bacterial cultures grown on TSA were scraped from the agar plate surface and collected at the bottom of the plate. The harvested bacteria was mixed with 1 ml of sterile 2% methyl cellulose, and surface sterilized rice seeds were placed in this solution for 30 minutes in order to coat the seed with the bacteria. Nontreated (control) seeds were coated with sterile methyl cellulose alone. Two weeks after seeding of rice in sterile soil, 500 J2 of *M. graminicola* were introduced around the roots. Seedlings were maintained for an addition 10 to 12 days under aerobic conditions to facilitate nematode penetration and gall formation. Seedlings were then harvested and galling severity evaluated through counting of individual galls.

**Attraction test**

Additional experiments were conducted to assess how one of the test bacteria (*Bacillus megaterium*) influences J2 attraction and penetration activities. The effect of *B. megaterium* inoculation of the rice root on *M. graminicola* host finding activity was evaluated using a 12x2x2 cm chamber filled with fine quartz sand. Two five-day old rice seedlings, either soaked in a *B. megaterium* suspension (10^7 cfu per ml) or in Ringer’s solution for 30 minutes, were planted at opposite ends of the chamber. Directly after planting, 1,000 J2 of *M. graminicola* were pipetted in the middle of the chamber, which was then covered with a plastic shell and sealed with Parafilm to prevent moisture loss. A treatment containing nematodes but no plants was included as a control. After one week, the chambers were disassembled and the wet sand was removed at 1.5 cm intervals, starting at each end and
moving towards the center, and transferred into separate vials. The nematodes were separated by wet sieving through a 250 µm sieve and collected on a 5 µm sieve, and counted using a stereomicroscope. The roots were stained with Acid Fuschin, and the number of invading J2 counted.

**Penetration test**

A penetration test was conducted with two week old rice seedling roots dipped in either a *B. megaterium* suspension containing 6 x 10⁶ cfu per ml or in Ringer's solution (control) for 30 minutes. Rice seedlings were then transplanted in 100 cm³ pots containing sterile 2:1 sand-soil mix soil, and the soil around the roots drenched with 5 ml's of washed bacteria cells or Ringer's. One week later, 900 J2 of *M. graminicola* were added around the roots, and one week after nematode infestation the roots were washed, stained with Acid Fuschin, and macerated in blender. The number of invading juveniles was counted under a stereomicroscope. All experiments were conducted twice.

**Results and discussion**

Four *Bacillus* species reduced nematode galling severity by 25 to 30% in the screening tests, indicating suppressive potential towards *M. graminicola* (Table 1). *B. megaterium* gave the most consistent results in repeated tests, and was thus selected for additional testing using root-dip inoculation. In the root dip tests, *B. megaterium*-treated roots had 40% fewer galls than the control roots (Figure 1). Besides reducing galling severity, smaller root-knot galls were observed on the *B. megaterium* treated roots compared with the nontreated roots.

Mode of action tests were performed for *B. megaterium* to quantify the extent to which this bacteria interferes with host finding and penetration activities by *M. graminicola*. Rice root penetration by J2 of *M. graminicola* was reduced 47% in roots treated with *B. megaterium* compared with nontreated roots (Figure 1). Treatment of the root system with *B. megaterium* also interfered with the host finding activity of *M. graminicola*, resulting in 55% fewer J2 in the roots and in the root-zone soil compared with the nontreated control (Table 2). No significant differences in nematode movement were detected for the control with no seedlings.

*B. megaterium* was also assessed for its facultative anaerobic capacity. In tests with an anaerobic jar (Anaerocult A, Merck), *B. megaterium* was observed to grow abundantly on TSA plates kept under anaerobic conditions for 48 hours (data not shown).

The capacity of *B. megaterium* to colonize the endorhiza, grow in an oxygen-stressed environment, and reduce nematode attraction and penetration make it well-suited for more intensive study in rice. One of the key criteria for successful endophytic biocontrol is whether the microbial antagonist can disrupt nematode development inside the root. This concern is particularly relevant to *M. graminicola* due to the fact that its egg sac is completely embedded inside the root cortex. Soriano et al. (2000) and Tandingan et al. (1996) measured 2 to 8 times, respectively, greater *M. graminicola* J2 density in rice roots subjected to mostly anoxic soil conditions compared with those where oxic conditions prevailed. Therefore, slowing *M. graminicola*’s development endorhizally is critical for reducing inoculum density in the roots.

A key challenge in designing endophytic biocontrol systems for rice root parasites is to understand how the durability and efficacy of the biocontrol organism is affected by soil flooding. External leakage of oxygen from the rice aerenchyma generates an oxic layer in the rhizosphere that is sufficient to support a number of aerobic microbial communities and processes on the root surface, even when bulk soil is anoxic (Briones et al., 2002; Liesack et al., 2000; Reichardt et al., 1997; Scheid et al., 2004). This oxic zone may serve as a buffer
against structural changes in endorhizal communities when soils are flooded. In fact, there is evidence that oxygen-rich microenvironments in the root aerenchyma facilitate colonization by some nitrogen fixing endophytic bacteria as soils become anoxic (Engelhard et al., 2000). Soil flooding seems to also enhance the functional efficiency of some root-associated bacteria. For instance, Engelhard et al. (2000) reported greater N-fixing efficiency of *Azoarcus* sp. in rice under oxygen-stressed environments. Similarly, low oxygen conditions in soil were reported to stimulate greater *Pseudomonas* colonization in the rhizosphere of tomato, resulting in enhanced fungal pathogen (Kim et al., 1996) and nematode (Siddiqui et al., 2003) biocontrol relative to that achieved in anoxic soil environments.

Table 1. Effect of endophytic bacteria on root galling of rice caused by *M. graminicola* in a screening test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. galls (per g fresh root)</th>
<th>% galling reduction (from control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>64.5 a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td><em>Bacillus circulans</em></td>
<td>48.1 a</td>
<td>25</td>
</tr>
<tr>
<td><em>B. laterosporus</em></td>
<td>47.6 a</td>
<td>26</td>
</tr>
<tr>
<td><em>B. megaterium</em></td>
<td>47.7 a</td>
<td>26</td>
</tr>
<tr>
<td><em>B. pumilus</em></td>
<td>45.2 b</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 2. Effect of *B. megaterium* on attraction of *M. graminicola* to rice roots, as measured by the number of J2 in roots and root-zone soil compared with that in non root-zone soil.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>root</th>
<th>root-zone soil</th>
<th>non root-zone soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>81.3 a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>132.2 a</td>
<td>32.1 a</td>
</tr>
<tr>
<td><em>B. megaterium</em></td>
<td>36.7 b</td>
<td>59.2 b</td>
<td>13.3 b</td>
</tr>
</tbody>
</table>

<sup>1</sup> Numbers followed by the same letter in a column are not significantly different at p< 0.05.

Figure 1. Effect of *B. megaterium* on *M. graminicola* penetration and root galling.
These studies suggest that there is considerable potential to exploit the conditions created by oxygen-limited soil environments for biocontrol of rice nematodes. We hypothesize that anoxic conditions in soil will increase the antagonistic potential of *B. megaterium* relative to its efficacy under oxic soil conditions. We base this hypothesis on i) the bacteria's aerobic/facultative anaerobic capabilities, ii) evidence of improved functional efficiency of root-associated bacteria under anaerobic conditions, and iii) possible competitive advantage at the onset of soil flooding for the target bacteria inoculated at high densities on the root.

Recent investigations on the role of rice bacterial endophytes in biological nitrogen fixation (Divan et al., 2000; Ladha et al., 1997) and in biologically controlling foliar pathogens of rice (Adhikari et al., 2001; Muhkopadhyay et al., 1996) illustrate the potential utility of this microbial community for plant growth- and plant health-promotion. However, there is little information on how the targeted bacterial inoculant reacts to the range of soil oxygen levels normally encountered in rice producing environments, nor how the endemic microbial community affects the introduced organism under different soil water environments. Understanding these influences is important for exploiting the full potential of plant growth/health-promoting bacteria in rice.

**Acknowledgements**

We wish to thank the US National Science Foundation for funding this research through the NSF International Research Post-Doctoral Fellowship Program.

**References**


Using flow cytometry for in situ monitoring of antimicrobial compound production in the biocontrol bacteria

*Pseudomonas fluorescens* CHA0

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² Institut de Microbiologie Fondamentale, Université de Lausanne, CH-1015 Lausanne, Switzerland

**Abstract:** *Pseudomonas fluorescens* strain CHA0 is able to protect plants against a variety of pathogens, notably by producing the two antimicrobial compounds 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT). The regulation of the expression of these compounds is affected by many biotic factors, such as fungal pathogens, rhizosphere bacteria as well as plant species. Therefore, the influence of some plant phenolic compounds on the expression of DAPG and PLT biosynthetic genes has been tested using GFP-based reporter, monitored by standard fluorometry and flow cytometry. In situ experiments were also performed with cucumber plants. We found that several plant metabolites such as IAA and umbelliferone are able to modify significantly the expression of DAPG and PLT. The use of flow cytometry with autofluorescents proteins seems to be a promising method to study rhizobacteria-plant interactions.

**Key words:** 2,4-diacetylphloroglucinol, biological control, flow cytometry, GFP, plant phenolic compounds, pyoluteorin.

**Introduction**

Certain strains of root-colonizing fluorescent pseudomonads are able to provide an efficient protection of crop plants against a variety of soil-borne phytopathogenic fungi, notably by the synthesis of extracellular antimicrobial secondary metabolites in the rhizosphere (Cook et al., 1993; Bloemberg et al., 2001). *Pseudomonas fluorescens* strain CHA0 produces the two well-characterised antimicrobial compounds 2,4-diacetylphloroglucinol (DAPG) (Keel et al., 1990) and pyoluteorin (PLT) (Maurhofer et al., 1994) which are major determinants of its biocontrol activity (Keel et al., 1992). There is evidence that their regulation in the rhizosphere is strongly affected by plant-derived factors (Notz et al., 2001).

Here we tested the influence of the three phenolic plant metabolites 3-indolacetic acid (IAA), umbelliferone, and resorcinol on the expression of the DAPG biosynthetic gene *phlA* and the PLT biosynthetic gene *pltA*, using reporter gene constructs with green fluorescent proteins (GFPs). Gene expression in liquid cultures assays was analyzed with a microplate reader as well as with flow cytometry. In order to study the DAPG and PLT regulation at a rhizosphere level, cucumber seedlings inoculated with CHA0-derivatives were grown in a hydroponic growth pouch system. Flow cytometry was then used to monitor in situ the expression of DAPG- and PLT-biosynthetic genes. This novel approach illustrates that the combined use of flow cytometry and autofluorescent proteins such as GFP is a promising method to monitor the expression of bacterial biocontrol genes in the rhizosphere and to study their regulation by biotic environmental factors.
Material and methods

**Assay for monitoring expression of GFP-based reporter fusions with a fluorescence microplate reader.**

_Pseudomonas fluorescens_ strain CHA0 and its derivatives carrying the different gfp-based transcriptional fusions were grown in 200 µl of OSGly medium without selective antibiotics using 96-well black microtitre plates with flat transparent bottom. For the assay, 10 ml of OSGly medium was inoculated with 20 µl of exponential-growth phase LB cultures of the bacterial strains diluted to an OD at 600 nm of 0.05, and when appropriate supplemented with IAA 500 µM, umbelliferone 100 µM or resorcinol 100 µM. For each treatment, six wells of the microtitre plate were then partly filled with aliquots of 200 µl of the respective bacterial culture. Cultures were incubated at 28°C with shaking at 180 rev min⁻¹. In all experiments, OD at 600 nm (growth) and green fluorescence (excitation at 480 nm and emission at 520 nm) was measured with a fluorescence microplate reader throughout the exponential and stationary growth phases.

**Assay for monitoring in vitro the expression of GFP-based reporter by flow cytometry.**

Bacterial strains were incubated overnight in 10 ml LB with the required antibiotic. The following day, cultures were transferred to fresh LB without antibiotic. When cells had reached the exponential growth phase, the OD at 600 nm was adjusted to 0.05 and 40 µl of this culture were inoculated in 20 ml OSGlycerol medium with phenolic compounds at the appropriate concentration. After overnight incubation at 28°C, 180 rev min⁻¹, each culture was diluted to a final OD at 600 nm of 0.01 to obtain the same cell number per sample (about 10⁷ ml⁻¹) for flow cytometry (FACS) analysis.

**Assay for monitoring in situ the expression of GFP-based reporter by flow cytometry.**

Bacteria and plants were grown in an artificial environment, an autoclavable plastic pocket, within which there is a filter-paper distributing equally the nutriment, and thus supporting root development. To provide sufficient nutrients to plants, the Knop nutrient solution was used for watering (Keel et al., 1989). On day 1, cucumber seeds (Cucumis sativus, cv. Chinese snake) were surface-disinfected for 10 min in a 4% NaClO solution and washed several times with desalted sterile water. Seeds were pre-germinated on soft agar (8.5 g/l) plates for 3 days at 24°C in the dark. On day 3, bacterial strains were inoculated in 10 ml NYB without antibiotic and incubated overnight at 30°C, 180 rpm. On day 4, cells cultures were centrifuged, washed twice with sterile water, and resuspended in 10 ml sterile water. The OD₆₀₀nm of the bacterial suspension was adjusted to 1.0. Three cucumber seedlings were then placed per growth pouch and watered with sterile 15 ml Knop solution containing 500 µM of IAA or 100 µM of umbelliferone. Each seedling was then inoculated with 1 ml of the bacterial suspension (GFP-reporter). Each treatment was prepared in triplicate. Growth pouches were wrapped in aluminum foil to protect roots from light, and incubated in a plant growth chamber (day: 16 h at 22°C, night: 8 h at 15°C, relative humidity 80%) for 5 days. At the end of the experiment, roots of each pouch were cut, weighed and placed in 10 ml sterilized filtered water in sterile 50-ml plastic tubes. Vigorous stirring (150 rpm) was performed for 20 minutes to remove bacteria cells from the roots, and 1 ml of the resulting suspension was taken to be directly analyzed by FACS where they were excited by an argon laser, emitting at a fixed wavelength of 488 nm. Flow cytometric total counts (FCTC) were determined as the total number of particles of a size and shape similar to bacterial cells by using side-angle light scatter (SSC) versus forward-angle light scatter (FSC) detector. GFP-positives cells were counted with the SSC versus green fluorescence (FL1) detector.
Results and discussion

In vitro effects of plant phenolics on the expression of DAPG and PLT biosynthetic genes

A survey of several plant phenolic compounds has been performed to test their ability to modify the expression of the DAPG and PLT biosynthetic genes cluster. *Pseudomonas fluorescens* CHA0 carrying GFP-based reporter fusions was grown in microtitre plates containing OSglycerol medium amended with different plant phenolic compounds. Differential DAPG and PLT gene expression was measured by a fluorescence microplate reader. We have found three interesting compounds namely IAA, umbelliferone, and resorcinol which had an effect on gene expression. The results (Figure 1) show that, under this artificial condition, the expression of the DAPG biosynthetic genes can be induced by IAA, and strongly reduced by umbelliferone. Moreover, the expression of the PLT biosynthetic genes is fully repressed by resorcinol.

![Figure 1. Effects of plant phenolic metabolites on (A) phlA'-gfp expression and (B) of pltA'-gfp expression in *P. fluorescens* CHA0. ● control; ▲ + IAA 500 µM; ■ + umbelliferone 100 µM; ● + resorcinol 100 µM. Strain CHA0 carrying the plasmid-borne fusions was grown in minimal medium OSglycerol in microplates; each point represents the mean ± standard errors of six replicate measurements.](image)

Using flow cytometry to monitor the effects of plant phenolics on the expression of DAPG and PLT biosynthetic genes in vitro

The following step was to evaluate the possibility of obtaining similar results when using a flow cytometry analysis. To induce changes in the expression of the DAPG or PLT biosynthetic genes, phenolic compounds were added as in the previous experiment. Cells were cultivated in OSglycerol supplemented with the phenolic compound. For the flow cytometry analysis, 1 ml was sampled from the culture, diluted to have an approximate concentration of $10^7$ cells per ml, and directly passed through the capillary of the FACS. The results obtained (Figure 2) show that flow cytometry is able to reproduce the same differential expression as observed with the fluorescence reader (Figure 1), and open the way to perform such experiments in situ. In brief, these results suggest that plant phenolic compounds have an effect on the expression of the biosynthesis of antimicrobial compounds, and therefore could affect the level of biocontrol provided by *Pseudomonas fluorescens* CHA0.
Using flow cytometry to monitor the effects of plant phenolics on the expression of DAPG biosynthetic genes in situ

In order to study the expression of the biosynthetic genes of antimicrobial compounds in situ, derivatives of *P. fluorescens* CHA0 harbouring GFP reporter-fusions were grown on cucumber roots, in an artificial growth pouch-system. Bacteria were harvest after 10 days of growth on roots, and analysed directly in a BD-FACSCalibur. It was still possible to observe the effect of the two utilised compounds on *phlA* expression, namely the induction by IAA and the repression by umbelliferone. In conclusion, our results show that flow cytometry offers new possibilities to study the regulation of bacterial biocontrol genes directly in the rhizosphere.
Acknowledgements

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References


Exploiting the potential of *Pasteuria penetrans* for biological control of root-knot nematodes

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Abstract: Commercial horticulture needs to develop pest management practices that are safe to the producer, consumer and environment. Root-knot nematodes are one of the production constraints for which a biological management strategy is urgently needed as a replacement for the relatively toxic nematicides. *Pasteuria penetrans* is an obligate bacterial parasite of these nematodes that could be deployed in the field by commercial growers and smallholders. Refinements in the system of mass-production of *P. penetrans* are dependent on a thorough understanding of the biology of the parasite and its host. This paper highlights technical issues concerning the production of *P. penetrans* spores that need to be addressed if this bacterium is to become an established biological control agent of root-knot nematodes.

Key words *Meloidogyne* spp, nematodes, vegetables, flowers, mass-production.

Introduction

Root-knot nematodes are one of the most intractable pest problems for intensive vegetable producers throughout the tropics and subtropics (Luc *et al.*, 2005). Diverse remedies are under investigation to meet contemporary demands of food retailers, consumers and environmental interests for safer, more sustainable production systems without compromising quality or profitability. These are based on combinations of cultural, physical and biological strategies. There are several biological control agents of nematodes but none has yet been developed that has achieved universal acceptability or adoption. Of those biological control agents under continued investigation, *Pasteuria penetrans* meets many requirements of an ideal organism but is perceived to lack promise as a biological control agent because of limitations in its mass-production. Dudutech is a biological control company in Kenya which has the capacity to invest in research and development. The company is responding to the need to develop biological control agents for the principal pest constraints of the commercial sector. For the root-knot nematodes (*Meloidogyne* spp.), Dudutech is producing *Pochonia chlamydosporia* and *Pasteuria penetrans*.

Technical progress

Experimental production under laboratory conditions can be quite variable (ranging from $10$ to $1000 \times 10^6$ spores per plant depending on the methodology and conditions: temperature, host, size of pot and numbers of nematodes used (Gowen & Channer, 1988; Hatz & Dixon, 1992).

Using the *in vivo* method of mass production on root systems of tomato plants similar to that described by Stirling and Wachtel (1980) large-scale production of *P. penetrans* in Kenya has been modest yielding $14 \times 10^6$ spores per plant (Rovesti 2005 unpublished). If accepted that a recommended field dose is $10^3$ spores per cm$^3$ incorporated to a depth of 15 cm (Trudgill *et al.*, 2000) the yields produced by this system need to be improved upon.

The Stirling and Wachtel (1980) *in vivo* production system requires host plants to be inoculated with spore-encumbered juveniles. The percentage of infection is related to the
number of spores per juvenile; yet the higher the spore attachment per juvenile fewer nematodes will invade, therefore, in finding a balance between invasion and infection most workers have adopted an attachment level of 6-10 spores per nematode as a "rule of thumb" estimate when preparing juveniles for use as inoculum. However, even when these criteria are met not all of the spore-encumbered nematodes that invade a root will become infected by Pasteuria because spore attachment does not always guarantee infection.

For nematodes, the major determining factor for development and fecundity is temperature. Tyler (1933) first examined the growth of root-knot nematodes in detail and introduced the term “heat units”. Stirling (1981) discussed the development of M. javanica and P. penetrans in terms of degree-days using 10°C as a thermal base temperature below which development of the organism(s) ceases. This thermal time concept allows the prediction of population development. It is said that the development of the parasite (Pasteuria) is “in synchrony with its host” this is true in the sense that the infected nematode undergoes its normal development to maturity within the same time frame as a healthy nematode. However, the development of spores of Pasteuria in infected females and that of egg production in healthy females is far from synchronous. Depending on the Meloidogyne species and temperature, eggs may be produced 20 days after invasion. Stirling (1981) reported that healthy M. javanica females commenced egg laying after a similar number of heat units had been accumulated regardless of temperature, with egg masses containing 100-300 eggs after 400 degree days at either 20°C, 25°C or 30°C. The minimum requirement for Pasteuria-infected females to contain predominantly mature endospores is 700 degree days at base 10°C accumulated at 30°C (35 calendar days). However at 20°C, a temperature less favourable to Pasteuria reproduction, maximum spore production may not be achieved in less than 100 days. At temperatures optimal to both organisms (25 – 27°C) spore production time is double that of the time to the start of egg laying, 42 days compared to 21 days respectively.

Darban et al. (2004) showed the importance of obtaining the correct balance between the maintenance of a healthy plant and the parasitic burden of Pasteuria-infected nematode females at temperatures favourable to the plant, nematode and hyperparasite. Also, Darban et al. (2004) showed that to obtain highest spore yields per female, the period of growth of the production plants should be for 12 weeks (or longer).

On the assumption that the longer production plants are grown the greater will be the numbers of spores, there is also a risk that non-infected nematodes in the system will produce a succession of nematode generations which could have a deleterious effect on the vigour of the production plant. This problem can arise when Pasteuria fails to infect some spore-encumbered juveniles at the start of a production cycle.

Pasteuria is dependant on the well being of its host - the nematode, which in turn requires a thriving host plant. Spore productivity requires a living system (host) and numbers will increase as long as the host nematode continues to feed and is receiving sufficient nutrients from the plant.

Under glasshouse conditions infected female nematodes were still increasing in weight after 88 days and contained 2.4 million spores/female (Darban et al., 2004). Further research might show that greater numbers of spores per female could be achieved by careful manipulation of the growing conditions, ensuring that the nematode burden on the plant does not lead to premature senescence.

Discussion

The COLEACP Pesticides Initiative Project (PIP) and the partnership of retailers and producers (EUREPGAP) which develops standards and procedures for agricultural producers
have been created to harmonise the regulations on pesticide usage. These European Union initiatives which require reduced levels of chemical inputs for commercial and export horticulture reflect public concerns on pesticide residues in fresh fruit and vegetables. Produce grown in or imported to Europe must now comply with these regulations. In cases where maximum residue levels (MRLs) are not available no residue levels are permitted. In view of this, all possible control strategies need to be exploited. The Pesticide Initiative Programme (PIP) was created to respond to the difficulties encountered by small producers from Africa, the Caribbean and Pacific. In Kenya, export horticulture is a major source of income, generating employment for 0.25-0.5 million smallholders. If this industry is to be maintained, all farmers will be obliged to adopt biological control agents such as *P. penetrans*.

Laboratory experiments done over several years have shown the difficulties and complexities of the *in-vivo* mass-production system of *P. penetrans*. This experience has provided the basis for scaling-up production and companies such as Dudutech (Kenya) Ltd. are now in the position to develop the potential for this biological control agent for use by flower and vegetable producers.

**Acknowledgements**

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**References**


Decayed *Armillaria mellea* fruiting bodies as source for potential biocontrol agents against root rot disease

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**Abstract:** *Armillaria mellea* is the causal agent of root rots of several perennial woody plants. On grapevine *A. mellea* infections cause reduction of plant vigor, poor grape quality and, in the last stage, the death of the plant. Chemical control is not effective and the available control measures are based on long rotations with non-susceptible crops. The use of biocontrol agents could be an additional tool to prevent infections in the new plantations. Several microorganisms were shown to be antagonistic to *A. mellea*, in controlled conditions both *in vitro* and *in planta*, but in the field their effect is usually weak and short lasting. To identify highly aggressive hyperparasites, 14 partially degraded *A. mellea* fruiting bodies were collected in infected vineyards in northern Italy (Trentino) and 109 microorganisms were isolated and identified. Their potential biocontrol activity against *A. mellea* was preliminary screened in vitro in dual culture on Petri dishes. Then the most effective microorganisms were tested on wood portions inoculated with the pathogen and then tested on *A. mellea* artificially infected strawberry plants. Filamentous fungi and bacteria were more frequent than yeasts on *A. mellea* fruiting bodies. *Aspergillus*, *Penicillium* and *Alternaria* species, even if effective in controlling the disease, were not selected because unsuitable as biocontrol agents. Few microorganisms (*Gliocladium* sp., *Trichothecium roseum*, *Cladosporium cladosporioides*, an unidentified bacterium, an unidentified *Actinomyces* and a sterile mycelium) showed a promising antagonistic activity against the pathogen.

**Key words:** root rot, grapevine, *Armillaria mellea*, biocontrol, antagonist

**Introduction**

*Armillaria mellea* is the causal agent of root rots of several perennial woody plants (Fox, 2000). On grapevine *A. mellea* causes reduction of plant vigour and poor grape quality (Guillaumin et al., 1982; Prospero et al., 1998). Grapevines can survive several years after the infection, but in the last stage, when most of the roots are killed by the fungus, the plant generally dies. *A. mellea* is a sapro-parasitic basidiomycete that can survive in the soil for long periods without the living host. The spread of the disease in the field is mainly due to rizomorphs that grow in the soil and colonise roots of closed by healthy plants. Basidiocarps sporadically appear in infected vineyards and basidiospores have only a marginal role in disease diffusion (Fox, 2000). Rotted roots residues or other woody material can act a substrate on which the fungus is able to survive for several years. Several studies have been done to find active chemicals against the disease, but none of them was found really effective (Cacioppo, 1987; Fox et al., 1994)), mainly because they cannot penetrate the rotted wood or roots where the pathogen lives. The only available measures to protect new plantations from infections originating from soil residues are on long rotations with non-susceptible crops. In Trentino region, Italy, *A. mellea* is an increasing problem on several crops (apple, plums, blueberry, etc.), but on grapevine damages are considerable (Sannicolo et al., 2002). This is due mainly due to the high value of land and products which incites growers to immediately replant single lost grapes or whole vineyards.
Long lasting antagonists in the soil could limit *A. mellea* growth, reduce the inoculum and therefore help in preventing new infections. Several microorganisms were shown to be antagonistic to *A. mellea*, in controlled conditions both *in vitro* and *in planta* (Dumas & Boyonoski, 1992; Fox et al., 1994; McQue, 1992; Raziq & Fox, 1999), but in the field their effect is usually weak and short lasting. Biocontrol agents can have different mechanism of action: antibiosis, competition for space and nutrients, resistance inductions, hyperparasitism. Hyperparasites or microorganisms that live by degrading *A. mellea* carpophores or rizomorphs can theoretically survive until the pathogen is present in the soil and therefore weaken or kill it.

Site of isolation and screening are critical steps in the discovery of microbial agents that can exert biological control of *A. mellea* at the root level. The objectives of this research were to determine the utility of isolating microorganisms from decayed fruiting bodies of the root rot pathogen and the usefulness of selecting potential BCAs using a three-steps screening procedure.

**Material and methods**

**Isolation of microorganisms**
In October 2004 several *A. mellea* infected vineyards were monitored to for presence of fruiting bodies of the pathogen. Fourteen partially degraded *A. mellea* fruiting bodies were collected, gently washed and crushed in sterile water. Microorganisms were isolated using serial dilutions of the crushed material on Potato Dextrose Agar (PDA), at 0 °C and divided in three groups: filamentous fungi, yeasts and bacteria.

**In vitro screening: dual culture and test on wood pieces**
The potential biocontrol activity against *A. mellea* of the isolated microorganisms was *in vitro* preliminary screened using dual culture. Each microorganism and the pathogen were inoculated on PDA in Petri dishes at a distance of 5 cm from each other (3 replicates) and maintained at 10°C. The radius, perpendicular to antagonist, (AC) of *A. mellea* colony was measured when the opposite side of the colony was at least 2.5 cm long (AD). Antagonistic efficacy of micro-organisms was evaluated as reduction of pathogen growth (AD-AC). Microorganisms were considered having a very good activity when \((AD-AC)*100/AD > 75%\).

The best organisms were selected for the successive experiments and identified. *Aspergillus*, *Penicillium* and *Alternaria* species were discarded, because unsuitable as BCAs, since they can produce toxins or they can be allergenic. The most effective microorganisms were tested on wood portions inoculated with the pathogen. The sterilised pieces of wood (cylinders 5 cm long and a diameter of 1 cm) were put in Petri dishes on PDA. The potential BCA and the pathogen were inoculated at the two ends. Five replicates were done. Biocontrol efficacy was calculated as: \((UAG-TAG)*100/UAG\), where UAG is untreated *A. mellea* growth in cm and TAG is the *A. mellea* growth when treated with the microorganism on the opposite site.

**In planta evaluation: greenhouse trials on strawberry**
The most promising microorganisms (with the highest biocontrol efficacy and fast growing) were tested on artificially *A. mellea* infected strawberry plants. Experiments were done in greenhouse controlled conditions (about 20 °C night temperature and 25° C day temperature) on potted strawberry plants, cv. Elsanta, grown on peat substrate. The artificial inoculation was done placing an *A. mellea* infected piece of wood in contact with the collar of the plant. Strawberry is used because it is indeed a host plant of *A. mellea* and symptoms appear quickly (less than six months), compared to grapevine, where it takes three-four years. Three replicates of three plants each in a suitable experimental design (fully randomised blocks)
were treated with 20 ml of culture broth and cells (incubated at 25 °C for 48 hours) of each microorganism. Water treated control was done. The percentage of infected plants (incidence) was calculated.

Statistical analyses of the data were performed using the Statistica 6.0 software (Statsoft). Incidence was arcsin transformed to obtain constant variance, before analysis of variance. Duncan’s test at $P=0.05$ was used to compare treatments.

**Results and discussion**

*Isolation of microorganisms*

In October 2004 fourteen partially degraded *A. mellea* fruiting bodies were collected in several highly infected vineyards. From serial dilutions of the suspension, 109 microorganisms were isolated and identified. Filamentous fungi (39.81%) and bacteria (55.6%) were more frequently isolated than yeasts (4.63%) from *A. mellea* decayed fruiting bodies.

*In vitro screening: dual culture and test on wood pieces*

Fifteen microorganisms (six bacteria, one yeast and eight filamentous fungi) were effective against *A. mellea* in dual culture test (Tab. 1).

<table>
<thead>
<tr>
<th>Code</th>
<th>Organism</th>
</tr>
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<tbody>
<tr>
<td>2932</td>
<td>Bacterium</td>
</tr>
<tr>
<td>2933</td>
<td><em>Gliocladium</em> sp. (cfr. solani)</td>
</tr>
<tr>
<td>2964</td>
<td><em>Engyodontium album</em> (Limber) de Hoog</td>
</tr>
<tr>
<td>2966</td>
<td><em>Cladosporium cladosporioides</em> (Fresen.) de Vries</td>
</tr>
<tr>
<td>2978</td>
<td>Mycelium sterile</td>
</tr>
<tr>
<td>3020</td>
<td>Bacterium</td>
</tr>
<tr>
<td>3022</td>
<td><em>Actinomycete</em></td>
</tr>
<tr>
<td>3025</td>
<td><em>Fusarium</em> Link ex Fr.</td>
</tr>
<tr>
<td>3027</td>
<td><em>Trichothecium roseum</em> (Pers.) Lik ex Gray</td>
</tr>
<tr>
<td>3031</td>
<td>Bacterium</td>
</tr>
<tr>
<td>3032</td>
<td>Yeast</td>
</tr>
<tr>
<td>3033</td>
<td>Bacterium</td>
</tr>
<tr>
<td>3040</td>
<td>Bacterium</td>
</tr>
<tr>
<td>3041</td>
<td>Bacterium</td>
</tr>
<tr>
<td>3049</td>
<td><em>Acremonium strictum</em> W. Gams</td>
</tr>
</tbody>
</table>

In the test on wood pieces *Gliocladium* sp. (2933), *T. roseum* (3027), *C. cladosporioides* (2966), an unidentified bacterium (2932), an unidentified Actinomycete (3022) and a mycelium sterile (2978) showed a promising antagonistic activity against the pathogen. Isolates coded 3020, 3022, 3040, 3041, 3049 were effective, but very poorly growing on liquid culture medium and were not selected for the *in planta* test.

*In planta evaluation: greenhouse trials on strawberry*

None of the isolates was able to fully control the disease on the inoculated plants. Only *Gliocladium* sp. (2933), *T. roseum* (3027) significantly inhibited *A. mellea* infections on strawberry plants and therefore can be considered promising biocontrol agents.
Conclusion

The results of the present work suggest that decayed fruit bodies of *A. mellea* can be a good source for isolating potential BCAs. Even if the two best isolates did not fully control the disease on artificially inoculated plants, with a suitable formulation or with repeated applications they could have practical value in the control of *A. mellea* root at the field level. Moreover the use of biocontrol agents could be an additional tool to be used in combination with other practices (rotations, biofumigations, etc.) to increase their efficacy in the new plantations.

Acknowledgements

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References


Influence on plant growth of *Glomus mosseae* BEG12, *Trichoderma viride* TV1 and *T. harzianum* T39 on grapevine in different environments

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**Abstract:** Root rot caused by *Armillaria mellea* s. s. and poor plant growth due to immediate replant is a serious and increasing problem in some grapevine growing areas in Trentino, Italy. Since arbuscular mycorrhizal fungi (AMF) are known to increase plant growth and resistance to certain wilt and root pathogens and some strains of *Trichoderma viride* and *T. harzianum* can act as biocontrol agents and have some effect on plant growth, their effectiveness on grapevine replant disease was evaluated both in field and greenhouse controlled conditions. The degree of plant growth enhancement caused by *Glomus mosseae* BEG12 (AMF), *T. viride* TV1 and *T. harzianum* T39 is strongly related to the different environments. Reduced effect of all the tested organisms was present in optimal growing conditions in greenhouse trials. The best approach to be used to study the environmental factors that influence the plant growth enhancement of the three organisms tested was discussed.

**Key words:** root rot, grapevine, *Armillaria mellea*, *Glomus mosseae*, *Trichoderma viride*, *Trichoderma harzianum*

**Introduction**

Root rot caused by *Armillaria mellea* s. s. and poor plant growth due to immediate replant is a serious and increasing problem in some grapevine growing areas in Trentino, Italy. The area involved in the study (140.37 hectare) is located in a valley called Piana Rotaliana. The high value of land and products causes growers immediately replant vineyards. The presence of *A. mellea* inoculum on the old vineyards roots that remain in the soil causes the infection of the new plants, whose symptoms appear after four-five years from plantation. Stunting, shortened internodes and reduced plant growth are all symptoms of replant disorders.

Arbuscular mycorrhizal fungi (AMF) are reported to increase of plant growth and resistance to certain wilt and root pathogens (Barea *et al*., 1998; Hassan *et al*., 1997). Some strains of *Trichoderma viride* and *T. harzianum* act as biocontrol agents and give some positive effects on plant growth (Bjorkman *et al*., 1998).

The aim of the research was to evaluate the efficacy of *Glomus mosseae* BEG12 (AMF), *T. viride* TV1 and *T. harzianum* T39 on grapevine replant disease and root pathogens.

**Material and methods**

**Greenhouse trials**

Experiments were done in greenhouse controlled conditions (about 20 °C night temperature and 25° C day temperature) on potted grapevines on peat substrate. The used variety was Pinot gris grafted on 101-14C (*Riparia X Rupestris*) rootstock. Three replicates of five plants each were weekly treated for three times with each treatment. Treatments started one week...
The applied treatments were: 10 g/plant *T. viride* TV1 (*Trichoderma viride* TV1, provided by Agribiotec, Italy), 10 g/plant *T. harzianum* T39 (Trichodex, provided by Intrachem Bio, Italy) and 20 ml/plant *Glomus mosseae* BEG12 (Endorize, provided by Agribiotec, Italy). Number of internodes, plant length and leaf surface was weekly recorded for 4 months.

*Field trials*

Experiments were done in 2000 and 2001 and data collected in 2001 and 2002. The treatments applied in each site in a suitable experimental design (randomised blocks) were listed in table 1. At least 50 plants for each replication were used. The dosage was the same used in greenhouses trials. Treatments were applied once, at plantation time.

### Table 1. Treatments in the different field sites and in greenhouse controlled conditions.

<table>
<thead>
<tr>
<th>Location</th>
<th>Treatments</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1a</td>
<td><em>G. mosseae</em></td>
<td>–</td>
<td>–</td>
<td>untreated</td>
<td></td>
</tr>
<tr>
<td>Site 1b</td>
<td><em>G. mosseae</em></td>
<td>–</td>
<td>–</td>
<td>untreated</td>
<td></td>
</tr>
<tr>
<td>Site 1c</td>
<td><em>G. mosseae</em> and <em>T. viride</em></td>
<td><em>T. viride</em></td>
<td>–</td>
<td>untreated</td>
<td></td>
</tr>
<tr>
<td>Site 2</td>
<td>–</td>
<td><em>T. viride</em></td>
<td>–</td>
<td>untreated</td>
<td></td>
</tr>
<tr>
<td>Site 3</td>
<td><em>G. mosseae</em></td>
<td><em>T. viride</em></td>
<td><em>T. harzianum</em></td>
<td>untreated</td>
<td></td>
</tr>
<tr>
<td>Greenhouse</td>
<td><em>G. mosseae</em></td>
<td><em>T. viride</em></td>
<td><em>T. harzianum</em></td>
<td>untreated</td>
<td></td>
</tr>
</tbody>
</table>

The data were subject to analysis of variance and means compared using Duncan’s multiple range test. Arc sin transformation was employed when necessary.

### Results and discussion

**Greenhouse trials**

Greenhouse trials result in no statistical difference among the treatments, even if some light effects on plant growth were seen on plants treated with *G. mosseae* and *T. harzianum*.

**Field trials**

*G. mosseae* was always effective on plant growth. Sites with similar environmental conditions give similar results (fig. 1), but the degree of efficacy in plant growth enhancement of *G. mosseae* differs from site to site. *T. viride* TV1 was effective on plant growth in site 2 (fig. 2), but gave no effect in site 1c (fig. 3).

Symptoms of *A. mellea* root rot were not present in the first and second year: it takes at least four-five years for *A. mellea* symptom expression on grapevine and many years are necessary for efficacy evaluation of treatments.

The effect on plant growth varies from site to site and seems to be absent if plants were grown in optimal conditions (greenhouse).

There are no satisfying *in vivo* model to test biocontrol agents (BCAs) and microbials with effect on replant disease on grapevine and this makes necessary the tests in natural conditions. The root rot disease (*A. mellea*) and the replant diseases in field are not uniformly distributed in field and large experimental areas are required. The application in commercial vineyards needs the use of registered BCAs.
Figure 1. *G. mosseae* increases plant vigour in the two locations in site 1. The locations have similar environmental conditions.

**T. viride (August)**

Figure 2. Increase of plant growth in site 2 due to *T. viride* treatment.

Figure 3. *T. viride* gave no effect on plant growth in site 1c. The effect of *G. mosseae* is present also in the second year.
Figure 4. *T. viride* gave no effect on plant growth in site 3. *G. mosseae* and *T. harzianum* significantly increase plant vigour.

It is necessary to define the right approach to the problem. Two approaches can be foreseen for the future: i) finding a new satisfying high throughput model for tests, that means to identify the possible factors that influences the effectiveness of the micro-organisms and to set up a controlled conditions method that include all these factors, but many factors have to be considered; ii) understanding the environmental conditions in which the micro-organisms are effective, that means the need of a strong interaction with computer science (new machine learning techniques, *i.e.* neural network approach), but a huge number of data are necessary.

**Acknowledgements**

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**References**


Molecular investigations of rhizobacteria-induced systemic resistance toward the root-knot nematode *Meloidogyne incognita* in tomato

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**Abstract:** A number of rhizobacteria have been shown to possess the ability to induce systemic resistance against the root-knot nematode *M. incognita* on tomato. Systemic resistance in these cases is characterised by a reduction in the numbers of galls and eggs produced per root-system in comparison to untreated root-systems. Differences between the bacteria strains with regards to their spectrum of action could be detected. However, the changes in plant metabolism that are linked to increased resistance are still unknown. In the present study, mRNA was extracted from the roots of induced and non-induced plants and the fragments of differently expressed genes enriched by subtractive suppressive hybridization. Results on the identity of these genes will be presented and possible benefits for agricultural practice will be discussed.

**Key words:** induced systemic resistance, rhizobacteria, *Meloidogyne incognita*, *Bacillus sphaericus* B43, *Rhizobium etli* G12, tomato, gene expression

**Introduction**

Biological control by rhizobacteria is a promising alternative methodology for use in the management of soilborne pathogens and pests. It is of particular interest for control of the root-knot nematode *Meloidogyne incognita*, where management is often limited to the use of broad spectrum soil fumigants. Rhizobacteria can have direct influence on the nematode or pathogen, but can also indirectly influence the plant to be protected by mechanisms related to induced systemic resistance (Kloepper et al. 1992). Bacteria mediated induced systemic resistance has been observed for example on raddish, tobacco, cucumber and *Arabidopsis* against both soilborne and foliar pathogens of fungal, bacterial and viral origin (van Loon et al. 1997, 1998). The “split-root-system” is usually used to separate pathogen and resistance inducer on one plant. One half of the root system is inoculated with the resistance inducing bacteria and the other half is inoculated with the pathogen (Zhou and Paulitz 1994; Liu et al. 1995).

Two rhizobacteria strains *Rhizobium etli* G12 (formerly called *Agrobacterium radiobacter* G12) and *Bacillus sphaericus* B43 have been demonstrated to reduce root penetration of the potato cyst nematode *Globodera pallida* (Racke and Sikora 1992). Both bacteria were later shown to induce systemic resistance in potato to the nematode (Hasky-Günther et al. 1998). In further studies, *B. sphaericus* B43 was shown to induce systemic resistance in tomato roots against the wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici* (Mwangi 2003) and toward the root-knot nematode *Meloidogyne incognita* (Hauschild et al. 2000). Conversely, *R. etli* G12 induced systemic resistance against *M. incognita* (Hauschild et al. 2004) but had no effect upon resistance to Fusarium wilt in tomato (Hauschild et al. 2001).
The reaction of the plant itself to the two rhizobacteria that induce systemic resistance against plant parasitic nematodes has not been investigated in detail. It is known however, that the application of \textit{R. etli} G12 and \textit{B. sphaericus} B43 does not lead to higher contents of PR-proteins as chitinases, β-1,3-glucanases or phenylalanin ammonia lyase in potato roots (Hasky-Günther 1996). The activity of glucanase, chitinase and peroxidase in potato roots after treatment with \textit{R. etli} G12 and in tomato after treatment with \textit{B. sphaericus} B43 also did not differ compared with untreated plants (Reitz et al. 2001; Mwangi et al. 2002). The accumulation, however, of “new” unidentified proteins was detected after bacteria application to potato roots (Hasky-Günther 1996).

In the present study, mRNA based methods were used in an attempt to detect differences in gene expression that might lead to differences in accumulation of defense-related or membrane proteins, secretion of cell-wall or soluble proteines, or secretion of metabolites involved in plant-parasite interactions.

\textbf{Material and methods}

\textit{Tomato cultivar}

The tomato cultivar “Hellfrucht Frühhstamm” susceptible to \textit{Meloidogyne incognita} was used in all experiments. Seeds were sown in a low-nutrient Klasmann seedling substrate (Klasmann-Deilmann, Germany) which was maintained moist in the greenhouse at 23 ± 5°C until germination. Seedlings were transplanted into 0.5 l pots containing Klasmann plant growth substrate after three to four weeks or when they had two differentiated leaves.

\textit{Bacterial inoculum}

\textit{Bacillus sphaericus} B43 and \textit{Rhizobium etli} G12 were originally isolated from the potato rhizosphere (Racke and Sikora 1992) and stored at -80°C in cryo vials (CryobankTM, Mast Diagnostica, Reinfeld).

The \textit{Bacillus} was cultured in liquid tryptone soya broth (Oxoid, GB) on a rotary shaker (100 rpm) for 24 h at 28 °C. The \textit{Rhizobium} was cultured in liquid King’s B Medium B (pH 5.8; King et al. 1954) on a rotary shaker (100 rpm) for 36 h at 28°C. The bacterial suspensions were centrifuged for 20 min with 4000 rpm at 20°C and resuspended in steril water.

The optical cell density of the bacterial suspensions were adjusted to \( \text{OD}_{560} = 2.0 \), representing approximately \( 1.57 \times 10^8 \) cfu ml\(^{-1} \) of \textit{B. sphaericus} B43 (Olzem 2001) and \( 1.0 \times 10^{10} \) cfu ml\(^{-1} \) of \textit{R. etli} G12 (Reitz et al. 2001).

\textit{Nematode inoculum}

The root-knot nematode \textit{Meloidogyne incognita} was obtained from a greenhouse stock culture on tomato. The eggs of the nematodes were extracted by the technique according to Hussey and Barker (1973). The second stage juveniles (J2) were obtained by placing egg suspensions in water for 10 day, during which time approx. 30 % of the juveniles hatched. The J2 were separated from the remaining eggs by sieving through filters as described by Oostenbrink (1960). The juvenile suspension was then adjusted to the required concentration with tap water.

\textit{Induced resistance split-root system bioassay}

Approximately 10 days after transplantation of the tomato plants into individual pots, or when they had three differentiated leaves, the plants were selected for the split-root bioassay. The plants were cut off at the base of the stem, and the hypocotyls were split longitudinally in two parts with a sterile scalpell to the lenght of approximately 3-5 cm. The parts of the hypocotyls were placed in in adjacent plastic pots (7 × 6 × 7 cm) filled with a mixture of sand and field soil (1:1, v/v) (modified after Fuchs et al. 1997) and were cultivated in the greenhouse for one week.
The split-root bioassay plant growing in one of the two pots were then drenched with 5 ml bacterial cell suspension. Sterile water was applied to the untreated root part in the other pot and was also used to treat the absolute control plants. Three days later *M. incognita J2* were infiltrated into the rhizosphere by drenching the soil surface with 5 ml suspension containing 1000 J2. Depending on the objective of the experiment, the nematodes were either infiltrated into the same pot as the bacteria or to the other pot. To extract plant RNA and to avoid contamination of the plant RNA with bacteria or nematode RNA, the bacteria and nematodes were inoculated together to the same pot and the other pot was left untreated.

To determine whether or not the rhizobacteria induced systemic resistance toward the nematode, the bacteria and the nematode were inoculated separately into different halves of the split-root pots system and so that the rhizobacteria inducer and the nematode pest remain spatially separated.

Root samples for RNA extraction were taken 3 and 6 days after bacteria inoculation. Tomato roots were carefully washed in running tap water and separated from the shoot. Roots were dried immediately with a paper towel, weighed, shock frozen in liquid nitrogen and stored until needed at -80°C. Root samples for the control of induced systemic resistance were taken 24 or 60 days after bacterization (data not shown).

**RNA extraction and Suppressive Subtractive Hybridisation**

From the untreated root part of the split-root system total RNA was extracted according to De Vries et al. (1986) in order to isolate mRNA (Nucleo Trap mRNA Kit, Machery-Nagel) as basis for the suppressive subtractive hybridisation (SSH, Diatchenko et al. 1996). This was conducted with the “PCR-Select cDNA Subtraction Kit” (BD-Biosciences Clontech, Heidelberg). cDNA-fragments of differentially expressed genes were selectively accumulated and amplified. After reverse transcription, cDNA-populations of plants which had been treated with different rhizobacteria were pooled, so that four different cDNA-populations resulted:

1. untreated plants, taken 3 days after bacteria inoculation (U3)
2. untreated plants, taken 6 days after bacteria inoculation (U6)
3. treated with *B. sphaericus B43* or *R. etli G12*, taken 3 days after bacteria inoculation (B+G)
4. treated with *B. sphaericus B43* or *R. etli G12* and *M. incognita*, taken 6 days after bacteria inoculation (BM+GM).

Two parallel SSH-procedures were conducted to isolate genes which were induced by bacteria alone (B+G) or in combination with nematodes (BM+GM).

During the first SSH the cDNA of bacteria treated plants, that is the cDNA that contains specific (differentially expressed) transcripts and is called „tester“(B+G), was compared with the reference cDNA „driver“, that is the cDNA of the untreated plants (U3). The second SSH was conducted analog with the first SSH, but with the cDNA of (BM+GM) as „tester“ and the cDNA of (U6) as „driver“.

The selected PCR-products were ligated into plasmid vectors and transformed in *E.coli DH5 α-T1R* (Invitrogen), and analysed with five restriction enzymes. Different clones were sequenzed by a commercial provider (MWG-Biotech, Germany).

**Results and discussion**

**Restriction enzyme analysis**

Five restriction enzymes were chosen to differentiate between the clones. With *EcoRI* the ligated PCR-product was cut out. Six clones had inside cutting sides of *EcoRI* but were not identical. Ten clones had no internal restriction sites for the used restriction enzymes *HindIII*, *XbaI*, *XhoI*, and *BamHI*. 
Two of them were identical (1-5 and 1-10). Four clones showed the same pattern of restriction sites for HindIII, XhoI, and BamHI and had an identical number of bases. Of the identical clones only one clone was choosen to be sequenzed. All other clones differed either in the number of bases or location of the restriction sites.

**Sequenzing**

After restriction enzyme analysis 19 different clones were detected. The 19 clones partially sequenced. The results of 12 sequenzed clones are shown in table 1.

Table 1. Identity of potential candidate genes with differential expression after induction by rhizobacteria or bacteria with nematodes.

[First number of clones : 1 = bacteria treated plants 2 = bacteria and nematode treated plants]

<table>
<thead>
<tr>
<th>Clone-nr.</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>no similarity</td>
</tr>
<tr>
<td>2-6</td>
<td>no similarity</td>
</tr>
<tr>
<td>1-2</td>
<td>similar sequence of <em>Arabidopsis</em> (unknown clone)</td>
</tr>
<tr>
<td>1-9</td>
<td><em>Lycopersicon esculentum</em> genomic sequence</td>
</tr>
<tr>
<td>2-1</td>
<td><em>Arabidopsis thaliana</em> mRNA</td>
</tr>
<tr>
<td>1-4</td>
<td><em>Solanum demissum</em> genomic sequence, <em>Nicotiana tabacum</em> poly(A)-binding-protein</td>
</tr>
<tr>
<td>1-5</td>
<td>similarity to binding-proteins and transporters</td>
</tr>
<tr>
<td>2-7</td>
<td><em>Solanum</em> sp. genomic sequence, wound-induced genes of potato</td>
</tr>
<tr>
<td>1-3</td>
<td><em>Lycopersicon esculentum</em> polygalacturonase</td>
</tr>
<tr>
<td>1-6 and 1-7</td>
<td><em>Lycopersicon esculentum</em> nitrate-transporter</td>
</tr>
<tr>
<td>2-B1</td>
<td><em>Lycopersicon esculentum</em> phenylalanin ammonia lyase (PAL)</td>
</tr>
</tbody>
</table>

Two clones showed no similarity to any presently known sequence, one clone from a bacteria treated plant and another from a bacteria and nematode treated plant.

Similarity to sequences of *Lycopersicon esculentum* genome or enzymes or transporters showed four clones. To other plants of the *Solanaceae* family showed two clones similarity, one of bacteria treated and the other one of bacteria and nematode treated plants. Three clones of bacteria treated plants showed similarity to binding-proteins and transporters. One clone of a bacteria and nematode treated plant showed similarity to wound-induced genes of potato. Another clone of a bacteria and nematode treated plant showed similarity to phenylalanin ammonia lyase.

**Discussion**

Although the true identity of the 12 sequenced clones has not yet been verified, the results give clue as to how the genes in tomato are differentially expressed following resistance induction by rhizobacteria either alone or together with nematode treatment. Because these results are only preliminary we can only speculate about the possible function of these transporters or enzymes.

A promising clone seems to be the one which shows similarity to phenylalanine ammonia lyase, as this enzyme is known to be associated with hypersensitive reactions of plants upon phytopathogen infection or wounding and also is involved in the phenylpropanoid metabolism leading to the synthesis of phytoalexins (Mayama et al. 1996).
Outlook

With the SSH technique used not all sequences shared in both tester and driver cDNA populations are effectively subtracted (Viebahn 2005). To confirm the results of the potential candidate genes with differential expression, Northern Blot hybridisations on total RNA of treated and untreated plants will be performed. This experiment could demonstrate which of the two bacteria strains induced the gene and if the presence of M. incognita is necessary for the induction of resistance.

References


Application of two biocontrol agents to potato roots – a risk assessment approach

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Abstract: The impact of the two *Verticillium* antagonists *Serratia plymuthica* HRO-C48 and *Streptomyces* sp. HRO-71 on the rhizosphere communities of potato was assessed in two consecutive field trials in Germany. The abundances of rhizobacteria as well as their proportion of *Verticillium* antagonists were determined. Additionally the culture-independent method of PCR-single strand conformation polymorphism analysis was applied to investigate culturable and non-culturable bacterial communities, respectively. No statistically significant differences between treatments and control plants were observed for the abundances of culturable rhizobacteria as well as for the proportion of in vitro *Verticillium* antagonists. The species composition of the non-target antagonistic bacteria did not differ between treated and untreated plants. By using the culture-independent method, only transient differences between the bacterial communities were observed with no correlation to the treatment. Computer-assisted comparison of total bacterial community fingerprints revealed only seasonal changes in the microflora. Nevertheless, the impact of treatment on the non-target microflora was lower than those of the growth stage of the plant or climatical factors.

Key words: risk assessment, biocontrol, potato rhizosphere, SSCP, *Serratia, Streptomyces*

Introduction

The soilborne fungus *Verticillium dahliae* Kleb. causes worldwide dramatic yield loss in many crops, e.g. strawberry, potato and oilseed rape (Tjamos, 2000). Biological control using antagonistic microorganisms became more and more attention as an environmentally friendly solution to control soilborne diseases (Whipps, 2001). The Gram-negative strain *Serratia plymuthica* HRO-C48 proved to be an effective antagonistic bacterium caused by its plant growth promoting and chitinolytic activity (Kurze et al, 2001; Frankowski et al., 2001), and was successfully developed as a product called Rhizostar® (produced by E-nema GmbH Raisdorff, Germany). The Gram-positive strain *Streptomyces* sp. HRO-71 was described as efficient biological control organism toward several plant pathogenic fungi by the production of siderophores, antibiotics, indole-3-acetic acid (IAA), and chitinases (Berg and Lüth 1999; Berg et al., 2001).

Although originating from the rhizosphere themselves, if applied in large densities into the rhizosphere of plants, the antagonists could be proved to be a threat to the indigenous microflora and their activity. Consequently, the impact of introduced microorganisms on the non-target root-associated microorganisms should be assessed. During the last decade, several molecular techniques have been developed, e.g. molecular fingerprint techniques on the basis of 16/18S rRNA and related genes like denaturing gradient gel electrophoresis (Muyzer and Smalla, 1998), terminal restriction fragment length polymorphism analysis (Liu et al., 1997) and single strand conformation polymorphism analysis (Schwieger and Tebbe, 1998), which enable the investigation of the whole microbial community.
The aim of our study was to analyse the impact of biological control agent (BCA) treatment on the indigenous bacterial communities by cultivation-dependent and cultivation-independent methods. Thus, the microbial rhizosphere community of potato (cv. Arkula and Celina) was investigated in two field trials in Germany following an application of two bacterial antagonists. A rifampicin resistant mutant of *Serratia plymuthica* HRO-C48 as well as the wildtype of *Streptomyces* sp. HRO-71 were applied.

**Materials and methods**

**Biological control organisms**
The strain *Serratia plymuthica* HRO-C48 (DSM 12502; Rhizostar®) and the strain *Streptomyces* sp. HRO-71 (DSM 12424; Rhizovit®) were originated from the rhizosphere of oilseed rape (Kalbe et al. 1996) and strawberry (Berg et al., 2000), respectively. For the preparation of the inoculums, the strain HRO-C48 was grown in nutrient broth (Oxoid, Hampshire, U.K.) while HRO-71 was cultivated in DSM medium 65, containing 4 g Glucose (Roth, Karlsruhe, Germany), 4 g Yeast extract (Sifin, Berlin, Germany), 10 g malt extract (Merck, Darmstadt, Germany), 2 g CaCO₃ (Roth) per liter (pH 7.2). After one day of pre-incubation in 100 ml medium, the BCAs were inoculated in a 10 liter Biostat B fermenter (B. Braun Biotech International, Melsungen, Germany) and grown at 30°C and 150 rpm for two (HRO-C48) and four (HRO-71) days.

**Plants and field trials**
Two consecutive field trials were carried out in Schwaan (Germany). In 2002 (trial I) cv. Arkula and in 2003 (trial II) cv. Celina was planted in blocks with five replicates. A rifampicin resistant mutant of HRO-C48 (C48-I/C48-II), the wildtype strain of *Streptomyces* sp. HRO-71 (71-I/71-II) as well as a water control (ctrl-I/ctrl-II) were applied. The potato tubers were dipped into the bacterial suspensions and water (control) for 15 to 20 min prior planting. The rhizosphere samples were taken three times a year in five week intervals from young plants, flowering plants and senescent plants, each treatment with five replicates. A sample consists of 5 g of roots with adhering soil from three plants.

**Abundances of bacteria and rifampicin resistant mutants**
The samples were pre-treated in a bag mixer in Stomacher bags (Interscience, St. Nom, U.K.) for 60 s, and were diluted in sterile PBS (130 mM NaCl, 10 mM natriumphosphate buffer). Determination of colony forming units (CFU) was performed by plating on R2A agar (Difco, Detroit, USA) containing 100 ppm nystatin (Fluka, Neu-Ulm, Germany). Rifampicin resistant mutants of HRO-C48 were reisolated on nutrient agar containing 100 ppm rifampicin (Fluka). Agar plates were incubated at 20°C for four days.

**Screening for antifungal in vitro antagonism**
Per treatment 150 randomly selected bacterial isolates were screened for antifungal *in vitro* antagonism towards *Verticillium dahliae* Kleb. by a dual culture assay. The assay was performed in 24-well-microtiter plates on Waksman agar. Material of one bacterial strain and 20 µl of *Verticillium* suspension (20°C and 130 rpm in Czapeck Dox medium) were incubated together at 20°C for four days. The formation of an inhibition zone of fungal growth around the tested isolate indicates an antagonistic activity of the strain. Statistically significant differences were determined by the Wilcoxon matched pairs signed rank test (p = 0.5 %).

**Characterization of the antagonistic strains**
The *Verticillium* antagonists were characterized by the molecular means of BOX-PCR fingerprints according to Rademaker and De Bruijn (1997). BOX-PCR fingerprints were clustered using GelCompar® software (version 4.1, Applied Math, Kortrijk, Belgium). The cluster analyses were used to calculate the diversity of antagonistic isolates, at 80% similarity
of fingerprints, applying the Shannon’s diversity index. Some antagonistic strains were identified (i) by their fatty acid methyl ester (FAME) profiles (Sasser, 1990), or (ii) by partly sequencing of the 16S rRNA gene followed by an alignment with reference sequences using the BLAST algorithm (Altschul et al., 1997). Based on all identified antagonists, the Simpsons index of diversity was calculated.

**Analysis of the community structure by single strand conformation polymorphism (SSCP)- analysis**

Microorganism pellets of the rhizosphere communities were obtained by serial centrifugation steps of the sample (5 g) at 450 x g for 5 min and 10000 x g for 20 min. The DNA was isolated using the FastDNA Spin Kit for soil and the Geneclean Turbo Kit (Qbiogene, BIO101® Systems, Carlsbad, USA). The procedure of single strand conformation polymorphism analysis (SSCP) was done according to Lieber et al. (2002). Bacterial communities were analysed using the universal eubacterial primer pair Unibac-II-515f/Unibac-II-927rP (Lieber et al., 2002) and the *Pseudomonas* specific primer pair F311Ps/1459rPs (Milling et al., 2004). The polyacrylamid gel electrophoresis was performed on a TGGE apparatus (Biometra, Göttingen, Germany) at 26°C and 400 V for 26.5 h using 8% (wt/vol) acrylamide gels. Conspicuous bands were eluted from the gel (elution buffer containing 0.5 M ammonium acetate (Sigma), 10 mM magnesium acetate (Sigma), 1 mM EDTA (AppliChem) and 0.1% SDS (AppliChem) for at least 4 h at 37°C) and identified by cloning and sequencing. DNA sequences from SSCP bands were compared with available sequences from database using BLAST-N program (Altschul et al., 1997) and the ARB software package (http://www.arb-home.de).

**Computer-analysis of SSCP gels**

The silver-stained SSCP gels were processed using GelCompar® software (Applied Math, Kortrijk, Belgium). After a procedure of gel normalization, which made it possible to compare band pattern of different gels, and background substraction similarity matrices were calculated using the band-based Dice similarity coefficient. Dendrograms representing the similarity of the microbial communities were constructed with the hierarchic cluster method of unweighted pair group method using average linkages (UPGMA).

**Results and discussion**

**Culture dependent analysis**

The abundances of culturable rhizobacteria ranged from log₁₀ 7.4 to 8.7 CFU g⁻¹ root fresh weight [rfw], and showed no statistically significant differences between the treatment samples and the control (α=0.1%). The establishment of the rifampicin resistant mutant of strain HRO-C48 could be confirmed. The strain was reisolated in numbers of log₁₀ 2.8 to 5.6 CFU g⁻¹ rfw.

A proportion of 6.1% of 2,897 tested bacterial isolates showed an antagonistic activity against *Verticillium dahliae* with no significant differences due to the treatments. However, Berg et al. (2005) found a higher antagonistic activity of bacteria against *Verticillium* (8%) in the potato rhizosphere, whereas results of Berg et al. (2002) showed an even higher antagonistic potential of the bacterial rhizosphere communities of strawberry (10.2%) and oilseed rape (8.9%).

Grouping of antagonistic isolates according to their genotypic fingerprints by means of BOX-PCR showed upto 27 different genotypic groups per sampling. Thereby no treatment or sampling specific BOX-patterns could be found. Based on these BOX fingerprints the Shannon’s diversity index was calculated, resulting in no significant differences between the treatments and the control. Representatives of the genotypic groups were selected for further characterization. Identified bacterial antagonists belonged to the phylogenetical groups: α-, β-
and γ-proteobacteria, high G+C Firmicutes, low G+C Firmicutes and the CFB group. The identification of antagonistic isolates from trial I (Tab. 1) revealed a dominance of members of the genus Pseudomonas with the species Pseudomonas putida as most abundant representative of this genus. This agrees with the results of Berg et al. (2005) who also found Pseudomonas species to be dominant in the potato rhizosphere and endorhiza.

Table 1: Diversity of identified antagonistic isolates from different treatments in trial I (2002).

<table>
<thead>
<tr>
<th>species</th>
<th>1st treatment</th>
<th>2nd treatment</th>
<th>3rd treatment</th>
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<tr>
<td>Acidovorax avenae</td>
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<tr>
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<td>Agrobacterium rubi</td>
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<tr>
<td>Azospirillum brasilense</td>
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<tr>
<td>Bacillus circulans</td>
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<td>Bacillus pumilus</td>
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<tr>
<td>Brevundimonas diminuta</td>
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<td>Chromobacterium violaceum</td>
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<td>Comamonas acidovorans</td>
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<td>Enterobacter cancerogenus</td>
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<td>Enterobacter pyrinus</td>
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<td>Flavobacterium johnsoniae</td>
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<td>Lechevalieria flava</td>
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<td>Micromonospora carbonacea</td>
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<td>Paenibacillus macerans</td>
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<td>Pseudomonas chlororaphis</td>
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<td>Pseudomonas corrugata</td>
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<td>Pseudomonas putida</td>
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<tr>
<td>Salmonella typhimarum</td>
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<td>Serratia plymuthica</td>
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<tr>
<td>Streptoverviciillium cinnamoneum</td>
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<tr>
<td>Simpson's diversity index</td>
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</table>

Culture-independent analyses
Using universal as well as Pseudomonas-specific primers, similar SSCP patterns in treated and un-treated plants were obtained. Single bands appeared with no correlation to the treatment. Computer-assisted comparison of SSCP fingerprints was carried out using the GelCompar® software. Thereby, fingerprints were grouped according to their similarity by calculating the Dice similarity coefficient. Similarities of community fingerprints ranged from about 70% between samples from different years and 82% – 91% between different plant growth stages. Most similar patterns (85%-100% similarity) were found between samples from different treatments. Figure 1 exemplarily shows the result of the cluster analysis of the SSCP fingerprints from the Pseudomonas communities of both trials.
Our results indicate that the community structure seemed to be less influenced by the treatment, rather than by seasonal shifts. Similar results were obtained in other culture dependent (Walsh et al., 2001) and culture-independent (Viebahn et al., 2003) studies after introduction of genetically modified bacteria on various plants. In these studies, seasonal shifts of community composition were detected but only minor, transient changes due to the applied beneficial microorganisms.

Using culture-dependent and culture-independent methods, no impact of bacterial treatments on the abundances, the antagonistic potential and the composition of the non-target rhizobacteria could be detected. Obviously, the establishment of the antagonistic strains does not represent a threat to the indigenous microflora of potato rhizosphere by competing other members of the community. Therefore, we do not recognize any definable non-target risk arising from using wildtype bacterial biocontrol organisms.

Acknowledgements

We thank Hella Goschke for technical assistance, Jana Lottmann for guidance on the GelCompar® software and Ralf Bastrop for help in 16S rDNA sequencing. This work was supported by Ministry of Consumer Protection, Food and Agriculture (No. 00HS029).

References


A chemo attraction in onion & citrus root exudates recognized by
*Ditylenchus dipsaci* & *Tylenchulus semipenetrans* in laboratory bioassays

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Introduction

A variety of chemical and physical cues are available to nematodes in their host seeking or food finding behavior. The primary mechanisms are believed to involve chemo tactic factors emanating from the host (Perry & Aumann, 1998). For plant-parasitic nematodes the major source of such factors is root exudates' components (Hale et al., 1987; Le Saux & Queneherve, 2002). Several responses to specific stimuli from root exudates have been detected since then: attraction and repulsion of *Meloidogyne incognita* (Diez & Dusenbery, 1989; Hale et al., 1987), characterization and partial purification of a kairomone that attracts *Heterodera schactii* (Ruhm et al., 2003), and responses of *Globodera rostochiensis* and *G. pallida* to potato root leaches (Devine & Jones, 2003; Rolfe et al., 2000).

Here we present a quantitative bioassay that translates preferences of field populations of two plant-parasitic nematodes, *Ditylenchus dipsaci* and *Tylenchulus semipenetrans*, observed *in vitro*, into relative attractiveness of sterile root exudates preparations (ORE – onion, and CRE – citrus) and their components.

The genus *Ditylenchus* contains diverse groups, mycophagous and phytophagous, suggesting multiple and varied adaptations. *Ditylenchus dipsaci* (Kuhn) Filipjev is polyphytophagous: approximately 500 plant species are known to be its hosts. Two races, sometimes sympatric, have been described in Israel (Aftalion & Cohn, 1990). The first infects canary grass but not onion and garlic. The second infects onion and garlic but not canary grass. A population of this second race, which causes severe damage to winter grown onion and garlic crops in Israel, has been used throughout these studies.

The citrus nematode, *T. semipenetrans*, a semi-endo parasite, is considered to be one of the most important nematode pests associated with citrus roots, which causes damage to the tree. In Israel, it was estimated by Cohn (Duncan & Cohn, 1990) that 4,000 second-stage juveniles (J2) per g root are the critical level for decline symptoms. Different management strategies have been recently suggested by Verdejo-Lucas and McKenry (Verdejo-Lucas & McKenry, 2004).

After reporting relative attractiveness of root exudate preparations from several plant species, and several types of onion, garlic and citrus rootstocks, we describe the preliminary characterization of a specific chemo attractant in root exudates from onion and a 'Troyer' citrange rootstock.

Results and discussion

Bioassays. The basic element consists of a pair-choice test conducted at room temperature (22-25°C) in a 9-cm diameter Petri plate containing autoclaved 1.5% Gelrite agarose in distilled water, spread into a thin film. To the cooling agar plate, 1ml of antibiotic solution
was added. Two sources, solutions of exudates or of treated exudates or controls, are spotted on the agar, 2.5 cm symmetrically from the center on the same diameter. A nematode suspension (usually 40 to 60 nematodes) is placed at the center of the plate after which it is covered. Collective mobility of the D. dipsaci is observable through a binocular microscope and counts are recorded after 30 and 60 min always, after 120 and 180 min sometimes. T. semipenetrans were incubated for, at least, 16 h at 25°C. One assay consists of several such pairings of sources performed simultaneously with five to seven replicate plates for every pairing. For comparisons of exudates from several plant species, all possible pairings of these source solutions with each other and with ORE or CRE (as a positive control) were included. In studies of various treated forms of ORE and CRE, it was more usual to include only pairings of those treatments with the 0.05 M CaCl2 buffer (as a negative control) or with untreated ORE or CRE (as a positive control).

**Relative attractiveness**

Nematodes are being offered a choice between two sources and their horizontal migration to one of the target areas (presumably up a diffusing gradient), and subsequently their movement around it or remaining stationary at it, is regarded as an expression of preference for the source at that target area over the alternative source at the other target area. Translation of observed preferences into relative attractiveness coefficients for the sources is based on a model for transitive preferences in pair-choice tests.

Onion (*Allium cepa*, 'White Lisbon') root exudates (ORE) was consistently and significantly much more attractive than the buffer control in all these assays. Exudates from oat cultivar Lodi, mustard cultivar Albatross and tomato cultivar Rehovot 13 were significantly more attractive than the buffer but less attractive than ORE; *Arabidopsis* cultivar Landsberg erecta, oil seed rape cultivar Cetes and wheat cultivar Bet Hashita were as attractive as the buffer, but canary grass and clover exudates were less attractive than the buffer and, therefore, were classified as repellent. No significant differences in relative attractiveness were detected among exudates from other two cultivars of onion (Texas Grano 502 and Granex Hybrid) and one cultivar of leek (Large American Flag), but exudates from one onion (Evergreen Long White Bunching) and one of the leeks (Broad London) were less attractive than ORE.

Host (onion) penetration study reveals that penetration preferences by *D. dipsaci* follow the same pattern as those predicted by relative attractiveness coefficients estimated in the bioassays.

Preliminary characterization of the chemo attractant from ORE, using the behavioral bioassay, demonstrated that it was stable to heat and to proteolytic enzymes, nonvolatile and water soluble with a molecular mass < 700 kD.

Root exudates from 'Troyer' citrange rootstock (*Citrus sinensis* L. x *Poncirus trifoliate*) (CRE) are consistently and significantly much more attractive than the buffer control in all these assays. Exudates from oat cultivar Lodi and wheat cultivar Bet Hashita are as attractive as the buffer, where tomato cultivar Rehovot 13 is significantly less attractive than the buffer and, therefore, is classified as repellent. No significant differences in relative attractiveness were detected among exudates from other two cultivars of citrus (Limeta and Volca), but exudates from one citrus (Trifoliata) was less attractive than CRE. Relative attractiveness is linear in relation to dilution exponent and therefore log-linear in relation to CRE concentration.

Preliminary characterization of the chemo attractant from CRE, using the behavioral bioassay, demonstrated that it is a water soluble and non-volatile component, stable to heat, lyophilization, proteolytic enzyme, and with a molecular mass < 3 kDa.
References


Heterogeneity of *Fusarium oxysporum* strains isolated as formae specialis *radicis-lycopersici*

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Abstract: Forl (*Fusarium oxysporum* f. sp. *radicis-lycopersici*) strains causing TFRR (tomato foot and root rot) isolated from greenhouses in The Netherlands were analyzed on the basis of the sequence of the IGS (intergenic spacer region) and of their virulence. Comparison of IGS revealed four clades of Forl strains. Strains shown to be most aggressive towards tomato were classified in four clades indicating that virulence is not a property of a certain group. Comparison of IGS sequences of Forl with those published in GenBank of Fol (*Fusarium oxysporum* f. sp. *lycopersici*) and of other eight formae specialis clustered Fol in groups with Forl strains. Strain PD87/245 is closely related to F.o. f. sp. *cyclaminis* on the basis of its IGS sequence. There is an indication that Forl strains differ in genetic origin.

Key words: *Fusarium oxysporum* f.sp. *radicis-lycopersici*, virulence, IGS, tomato foot and root rot

Introduction

The species *F. oxysporum* includes many plant pathogens that cause diseases on a broad range of agricultural and ornamental plants. *F. oxysporum* is assigned to the Fungi Imperfecti since no sexual cycle has been observed. So, the species concept for *F. oxysporum* is based on formae specialis. Non-pathogenic strains of *F. oxysporum* can not be described in the terms of formae specialis, since no host plant is known for them. The various pathogenic isolates of *F. oxysporum* are classified into more then 150 formae specialis with names reflecting susceptible plant species. The formae specialis system has no reliable predictive value, since it was shown that some pathogenic *F. oxysporum* strains can infect non-host plants (Baayen, et al., 2000).

Intensive molecular studies in phylogeny of *F. oxysporum* complex revealed both monophyletic and polyphyletic formae specialis (O’Donnel et al., 1998). Forl is pathogenic on tomato and causes foot and root rot. The strains of this formae specialis have a relaxed host range (Menzies et al., 1990) and might vary in aggressiveness of plant infection. These variations can be explained by genetic diversity of strains described as formae specialis *radicis-lycopersici*.

The aim of present work was to evaluate the diversity of Forl strains on the basis of IGS sequence analysis and to compare our model pathogen Forl ZUM2407 (Chin-A-Woeng et al., 1998) on the basis of IGS sequence and on its virulence on tomato plants with twelve strains assigned as Forl isolated from greenhouses in The Netherlands.
Material and methods

Strains, Media
Strains used in this study are listed in the Table 1. Fungal strains were grown at 28°C on PDA (potato dextrose agar) (Difco Laboratories, Deitroit, USA). Czapek-Dox broth (Difco Laboratories, Deitroit, USA) was used to obtain microspores of Forl strains.

DNA techniques
Fungal strains were grown on sterile filter paper placed on PDA agar. Filter paper with fungal hyphae was collected and ground in liquid nitrogen. DNA was isolated from pulverized fungal biomass using the Nucleon Phytopure kit (Amersham Biosciences GmbH, Freiburg, Germany).

Intergenic Spacer fragment of rDNA was amplified with the primers CNL12 and CNS1. The nucleotide sequence of the PCR fragments was determined by ServiceXS (Leiden, The Netherlands) using primers CNL12 and U49:65 (Cai et. al., 2003). Obtained chromatograms were normalized and exported to FASTA format with Chromas 1.45 program and assembled with DNAMAN 4.0 Software.

Sequence alignment was performed using CLUSTALW 1.8 program (Thompson et al., 1994). For construction of the consensus tree, bootstrapping was performed with 1,000 data sets and unweighted pair group and visualized with TreeView(Win32) software(version 1.6.6. Copyright® Roderic D.M. Page 2001).

Pathogenicity tests in gnotobiotic system and potting soil
Gnotobiotic system: sterile tomato seedlings cultivar Carmello (Syngenta) were cultivated under gnotobiotic conditions as described by Simons et al. (1996). After one week of growth plants were inoculated with fusaria spores (2x10^6 spore/kg sand). Diseased plants were scored after 4, 5, 6 and 7 days after spore inoculation. Each treatment consisted of 20 plants.

Potting soil: non-sterile tomato seeds were sawn in potting soil mixed with spore solution (2x10^6 spore/kg soil). Diseased plants were scored after 1, 2 and 3 weeks of cultivation. Each treatment consisted of 96 plants.

Results

Virulence assay
Virulence of fusaria isolates was tested in both gnotobiotic and non-sterile potting soil systems. Five isolates C63F, FORL MDI040126018, FORL 2037, FORL PD20006163 and FORL PD95/1187 caused highest levels of disease (78-85% sick plants) in both systems (Fig. 1). The other eight isolates also caused disease, but the percentage of sick plants was less than 50% (data not shown).

IGS sequences
Comparison of IGS sequences of our 14 strains with the IGS regions of 20 other strains from species F. fujikuroi, F. thapsinum and F. oxysporum with 8 different formae specialis gave a strongly supported (100%) ingroup comprising of F. oxysporum strains (Fig. 2). Earliest diverging lineage comprises of two Forl strains (bootstrap 82%). Cucumber pathogen Forc V03-2g is nested near Forl (clade3)-Fol cluster. This cluster is not highly supported (bootstrap 60%), but the distance between Forl strains of clade 3 and Fol strains is quite close. The Majority of Forl strains are nested (bootstrapping 98%) in clade 1 together with Fol strains, showing close relationship of the strains from two different formae specialis. Strains Forl PD20006163 and MDI031216059 form distinct clade with high bootstrap support (99%). Forl strain PD 87/245 is nested in poorly supported cluster (67% of
bootstrapping) with distantly related strains from four formae specialis (*cyclaminis, dianti, fragariae* and *cucumerinum*).

Table 1. Strains used in the study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Source</th>
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<tr>
<td>Forl MDI04012601</td>
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<td>Forl MDI031216059</td>
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<td>Forl C63F</td>
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Fig. 1. Virulence of most aggressive *Forl* strains tested in potting soil.

**Discussion**

TFRR was first reported in Japanese greenhouses on tomato as a new race (J3) of *Fol* (Sata and Araki, 1974). Research done on causal agent of TFRR allowed to classify this pathogen as new formae specialis *radicis-lycopersici* that causes foot and root rot and cannot infect tomato vascular system. (Jarvis and Shoemaker, 1978). It was shown that *Forl* strains can damage the root system of plants from families Solanaceae, Leguminosae, Cucurbitaceae and
Chenopodiaceae (Menzies et al., 1990). The wide range of putative host plants implies a high diversity inside of this formae specialis.

Fig. 2. Phylogenetic tree based on the IGS sequence comparison of 34 strains from 3 related species. *Fusarium oxysporum* species is presented as 9 different formae specialis. The numbers at the nodes represent bootstrap support >50% from 1,000 replications. The most virulent strains are marked by black arrows. Strains sequenced in this study are underlined.

We used the IGS sequence, known as a variable region, for comparison of *F. oxysporum* strains causing foot and root rot of tomato. Analysis of the *Forl* strains on the basis of IGS sequence revealed 4 clades. The five most aggressive strains were distributed over four clades indicating that virulence is not a property of a certain phylogenetic group of *Forl* (Fig. 2). The majority of the strains were clustered in clade1 together with our model pathogen ZUM2407, showing that this strain is a typical representative of *Forl*, not only based on the virulence but also on the IGS sequence (Fig. 2). Interestingly, clade 1 includes also *Fol* strains, that are closely relative to the *Forl* subclade. The same can be said on a clade of *Fol* strains nested near clade 3. In both cases *Fol* and *Forl* strains share similarity on IGS sequences and might have the same phylogenetic origin. Comparison of our *Forl* strains with representatives of other formae specialis showed that PD87/245 is distant from major clades of *Forl* strains.

IGS comparison of *Forl* strains revealed pronounced differences between *Forl* strains. These differences can imply different genetic origin of the strains and provide a reasonable explanation for the wide host range shown for this formae specialis of *F. oxysporum*. 
Acknowledgements

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References


Transcription factors in roots and shoots of Arabidopsis involved in rhizobacteria-induced systemic resistance

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Abstract: Plants possess inducible resistance mechanisms through which they can regulate their defense response to pathogen attack. Colonization of Arabidopsis thaliana roots by non-pathogenic Pseudomonas fluorescens WCS417r bacteria triggers a jasmonate- and ethylene-dependent induced systemic resistance (ISR) that is effective against a broad range of foliar pathogens. In the roots, the transcriptional activity of a large number of genes is altered upon colonization by WCS417r. To investigate the role of WCS417r-responsive, root specific genes in ISR signaling, we screened T-DNA insertion lines of a subset of these genes. Bioassays revealed that AtMYB72, a transcription factor gene specifically induced in the roots upon colonization by WCS417r, is essential for activation of ISR. The myb72 knockout mutant was incapable of mounting WCS417r-mediated ISR against the challenging pathogens Pseudomonas syringae pv. tomato DC3000 and Hyaloperonospora parasitica. Analysis of AtMYB72 gene expression revealed that ethylene is an important regulator of AtMYB72. This was supported by the finding that AtMYB72 was found to physically interact with the ethylene-regulatory protein EIL3 in a yeast two-hybrid assay. Transcript profiling revealed that ISR-expressing leaves are primed for augmented expression of predominantly jasmonate- and ethylene-responsive genes. Promoter analysis of these primed genes showed overrepresentation of an AtMYC2 binding motif, suggesting a regulatory role for this transcription factor in ISR. Further evidence for the involvement of AtMYC2 in ISR arose from bioassays showing that AtMYC2 knockout mutants were not able to show ISR after root colonization by WCS417r, while their level of basal resistance was comparable to that of wild-type Col-0.

Key words: Arabidopsis thaliana, Pseudomonas fluorescens, induced systemic resistance, transcription factor, AtMYB72, AtMYC2

Introduction

The direct surrounding of plant roots, the rhizosphere, is a very nutrient-rich habitat that provides a niche to numerous micro-organisms. Next to pathogens, also many fungi and bacteria with properties beneficial for the plant are present. Mycorrhiza and Rhizobia are well known examples of organisms that stimulate plant growth, either by enlarging the surface for nutrient uptake, or by enhancing the availability of nitrogen, respectively. Other beneficial micro-organisms help the plants in their defense, either by hampering growth and development of pathogens due to e.g. competition for nutrients or the secretion of antibiotics, or by stimulating the resistance mechanism of the plants themselves (Van Loon et al., 1998). Selective, non-pathogenic, species of Pseudomonas are a well known example of a group of organisms that lead to this state of induced systemic resistance (ISR) of the plant after colonization of the roots.

Research on mutants of Arabidopsis thaliana showed that responsiveness to the plant hormones ethylene (ET) and jasmonic acid (JA), and a functional NPR1 protein (non-expressor of PR-proteins) are essential for ISR (Pieterse et al., 2002). To identify genes that
are associated with ISR, transcriptome analysis of both root and foliar tissues of plants of which the roots were colonized by *Pseudomonas fluorescens* WCS417r was performed using Affymetrix GeneChips and compared to tissues of control-treated plants (Verhagen et al., 2004). This approach led to the identification of a MYB transcription factor in the roots and a MYC transcription factor in the systemic tissue that both seem to play a key role in the transduction of the ISR signal that is induced upon root colonization.

### Material and methods

**Cultivation of plants and induction of ISR**

Cultivation of *Arabidopsis thaliana* accession Col-0, the T-DNA-insertion knockout *myb72* and the *AtMYC2* mutant *jin1-2* (Berger et al., 1996), and induction of ISR by non-pathogenic *Pseudomonas fluorescens* WCS417r bacteria was performed as described by Pieterse et al. (1996).

**Pathogen bioassays**

Bioassays in which the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst DC3000*) was used for challenge inoculation, were performed as described by Pieterse et al. (1998). For *Hyaloperonospora parasitica* bioassays 3-week-old Arabidopsis Col-0 and *myb72* plants were misted with a conidiospore suspension of *H. parasitica* WAC09 containing 7.5 x 10⁴ conidiospores per ml. Inoculated plants were maintained at 17°C and 100% relative humidity for 24 hours. Subsequently, humidity was lowered to 70% to reduce direct effects on plant development and to reduce the chance of secondary infections by opportunistic pathogens. Seven days after challenge inoculation humidity once again was raised to 100% to enable *H. parasitica* to form sporangiophores. Disease symptoms were scored 9 days after inoculation. Quantification of callose deposition was performed following the method described by Ton et al. (2005).

**Yeast two-hybrid screen**

Constructs for yeast two-hybrid analysis were generated using vectors pDEST™32 and pDEST™22 (Invitrogen). Full-length cDNA inserts of *AtMYB72* and *AtEIL3* were introduced using GATEWAY™ technology (Invitrogen), following manufacturers instructions. The yeast two-hybrid screen was performed essentially as described by James et al. (1996).

### Results and discussion

**AtMYB72, a WCS417-resistant, root-specific gene required for ISR**

Analysis of changes in the transcriptome of Arabidopsis roots in response to colonization by ISR-inducing WCS417r bacteria revealed 97 genes that showed a locally altered expression in the roots (Verhagen et al., 2004). To investigate the possible involvement of these genes in ISR signaling, we started to systematically analyze knockout mutants of these genes for their ability to express WCS417r-mediated ISR. Out of 11 knockout mutants tested, one knockout mutant with a T-DNA insertion in the *AtMYB72* gene, which is significantly up-regulated in the roots upon colonization by WCS417r, was identified as unable to mount ISR against *Pst DC3000* in response to colonization of the roots by WCS417r.

To investigate whether *AtMYB72* is involved in ISR against other pathogens as well, bioassays with the oomycete *H. parasitica* were performed. After germination from spores, this pathogen forms an appressorium from which a penetration hyphae invades the leaf tissue in between epidermal cells. Successful invasion allows *H. parasitica* to colonize the leaf tissue and eventually form sporangiophores that appear from the leaf surface. Table 1 shows that WCS417r-mediated ISR against *H. parasitica* significantly reduced disease symptoms in wild-type Col-0 plants. However, in *myb72* mutant plants, ISR against *H. parasitica* was
completely abolished. Induced resistance against *H. parasitica* has been shown to be associated with priming for enhanced callose deposition at the site of penetration (Kohler et al., 2002; Ton et al., 2005). ISR-expressing Col-0 plants also show an enhanced induction of callose formation. However, in mutant *myb72* plants, this enhanced callose formation could not be detected. Together these results indicate that AtMYB72 is a component of the ISR signaling pathway that is essential for the induction of ISR against different pathogens.

<table>
<thead>
<tr>
<th>Increasing disease severity class</th>
<th>Relative disease rating¹</th>
<th>Ctrl</th>
<th>WCS417r</th>
</tr>
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<tbody>
<tr>
<td>Col-0</td>
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</tr>
<tr>
<td>1</td>
<td>60.5</td>
<td>70.1</td>
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<td>2</td>
<td>10.8</td>
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<td>3</td>
<td>23.3</td>
<td>22.2</td>
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<tr>
<td>4</td>
<td>5.5</td>
<td>1.7</td>
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<tr>
<td>myb72</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>53.6</td>
<td>52.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.8</td>
<td>11.3</td>
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<tr>
<td>3</td>
<td>27.5</td>
<td>27.4</td>
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<tr>
<td>4</td>
<td>11.0</td>
<td>8.4</td>
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</tr>
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</table>

¹ The distribution over the disease severity classes was significantly different from the control in Col-0, but not in *myb72* (Chi-square, α = 0.05).

**AtMYB72 expression is regulated by ET**

Using the ET-insensitive mutant *ein2-1* (Guzmán and Ecker, 1990), Knoester et al. (1999) demonstrated that the induction of WCS417r-mediated ISR requires ET-signaling at the site of application of the inducer. To investigate whether WCS417r-induced expression of *AtMYB72* in the roots is also regulated by ET, transcript accumulation of this gene was studied in WCS417r-treated *ein2-1* plants. The absence of induced *AtMYB72* mRNA levels indicated that ET indeed has a regulatory function in *AtMYB72* induction. Further evidence for the involvement of this hormone in *AtMYB72* expression came from the observation that *AtMYB72* transcript levels accumulate after treatment with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC).

**AtMYB72 interacts with EIL3 in vitro**

The yeast two-hybrid system is a frequently used molecular genetic tool to study *in vitro* protein-protein interactions. The set-up of this system enables yeast to grow on selective medium only if reporter-genes are activated by the transcription regulation protein GAL4. To do this, the DNA-binding domain (BD) and the activation domain (AD) of GAL4 are needed in close proximity of each other. Fusing two proteins of interest each to a different domain of GAL4 will lead to expression of downstream reporter genes only if the two domains are brought together due to interaction of the two proteins to which they are fused. Using this technique AtMYB72 was found to physically interact with the EIN3-like protein EIL3. EIN3 and its paralogs, the EIN3-like proteins EIL1, EIL2 and EIL3, are nuclear transcription factors that bind to the promoters of ET-responsive genes, such as *ERF1*, and initiate a transcriptional cascade leading to the regulation of ET target genes (Solano et al., 1998). These results again suggest a role of the ET signaling pathway in the regulation of AtMYB72 function.
**Priming of JA-dependent responses during ISR is regulated by AtMYC2**

Transcript profiling of ISR-expressing Arabidopsis leaves revealed that the onset of ISR is not associated with detectable changes in gene expression. However, upon pathogen attack, a large set of predominantly JA/ET-regulated genes showed a potentiated expression pattern. Evidently, ISR-expressing plants are primed for augmented expression of pathogen-inducible genes, which might allow the plant to react more effectively to a broad spectrum of pathogens (Verhagen et al., 2004). To further elucidate this WCS417r-induced priming-phenomenon the promoter regions of genes that showed an augmented expression in response to treatment with methyl jasmonate (MeJA) were scanned for the presence of common binding sites of regulating transcription factors. This analysis led to the finding that binding motifs for AtMYC2, a transcription factor protein known to be responsive to JA (Lorenzo et al., 2004) were overrepresented, suggesting that AtMYC2 plays an important role in ISR signaling.

To further investigate the role of AtMYC2 in ISR, a mutant of AtMYC2, jin1-2 (Berger et al., 1996) was tested for its ability to show WCS417r-mediated ISR against *Pst* DC3000. While WCS417r-treated Col-0 plants showed a significant reduction in the percentage of leaves with symptoms, jin1-2 did not, indicating that jin1-2 is impaired in its ability to express ISR. Hence, AtMYC2 is of crucial importance for ISR. Detailed analysis of *AtMYC2* expression in ISR-expressing tissues revealed that *AtMYC2* mRNA levels are significantly higher in systemic tissues of WCS417r-induced plants, suggesting that enhanced levels of the AtMYC2 transcription factor are responsible for the augmented expression of JA-responsive genes as observed in ISR-expressing plants.

**Acknowledgements**

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Concepts of multitrophic interactions

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Abstract: The development of the concept of multitrophic interactions has been strongly driven by aboveground biocontrol research. Biocontrol studies below ground have contributed far less to ecological concept development. Here, I discuss some of the principles of the multitrophic interactions concept. Then, I argue that above and belowground biocontrol studies should become more integrated, as plants are exposed to both above and belowground enemies at the same time. Control of one natural enemy, either above or below ground, may influence plant exposure to other enemies in the same or in the opposite subsystem.

Key words: above-belowground interactions, natural ecosystems, biocontrol, agriculture

Introduction

Ecological concepts are usually developed using visible and easily manipulated model systems. The unfortunate fate of ones working in more complex systems is then to test if these relatively simple concepts also hold in harsher conditions. Considering the difficulty of experimental studies with soil organisms, it is not surprising that the concept of multitrophic interactions has been strongly developed for aboveground plant-herbivore-carnivore systems. In particular, many of these studies have focused on how crop plants may deal with their aboveground invertebrate enemies. Nevertheless, that work leaves us with a number of questions. Is this multitrophic interactions concept generally applicable? Do multitrophic interactions apply equally well to pathogens as to invertebrates? Is recruitment of antagonists as reliable in the soil as above ground? Has the breeding of crop plants for fast growth, high yield and nice taste resulted in a trade-off with the capacity of direct defense, so that we are underestimating the role of direct defense in wild plants? How do multitrophic interactions relate to biodiversity and how does biodiversity relate to multitrophic interactions? Are below and aboveground multitrophic interactions linked? These are obviously too many questions to be answered in one contribution, but they may serve well as a kick-off for an introduction paper on concepts in multitrophic interactions for a symposium volume on multitrophic interactions in biocontrol. I will limit myself to discuss some aspects of multitrophic interactions, trade-offs between different plant defense options, and the considering of above and belowground multitrophic interactions as one integral aspect. Even when carried out in isolation from aboveground interactions, biocontrol studies focusing on soil parasites and pathogens may benefit from a more conceptual multitrophic interactions approach.

Concepts, hypotheses and paradigms

Scientific research develops through observations and the testing of hypotheses by experiments. The formulation of concepts can help in structuring observations (Figure 1). For example, when crop protection through multitrophic interactions between plants, herbivores
and the natural enemies of the herbivores is considered as a concept, the concept structures the observations, for example that the control of plant parasitic nematodes is correlated with the presence of antagonistic microorganisms. The causality of the observation (microbes control nematodes) needs to be verified by experiments (crops produce more with nematodes and antagonistic microbes together than with nematodes alone), which results in a theory (nematode control strongly depends on the presence of specific microorganisms in the soil). This theory supports the development of a paradigm, namely that crops can be protected against natural enemies by biocontrol. Paradigms can shift, for example the awareness that crop enemies can be controlled by natural enemies makes that chemical crop protection has been, at least partly, replaced by biological control. In fact, although not being aware of this, scientific research develops from conceptual approaches leading to paradigms (Figure 1). Concepts can develop and paradigms can shift, as a consequence of progression of insight.

**Direct and indirect controls and their interactions**

Biological control of aboveground insect pests has developed strongly according to a conceptual approach, from studies on bitrophic to tritrophic to multitrophic interactions. This development has also taken place in studies on biological control of nematodes and soil-borne pathogens, however, much less explicitly focussing on concept development. In a multitrophic context, the control of natural enemies can be via bottom-up (resource based) and top-down (natural enemy based) interactions. For crops, this means that natural enemies can be controlled both through resistance breeding (bottom up) and biocontrol (top down). According to the concept of multitrophic interactions, there may be trade offs between direct (bottom up) and indirect (top down) enemy control processes, as well as within these control processes. For example, plants may produce trichomes against aboveground natural enemies, but these trichomes can hamper ant movements when chasing away aboveground herbivores (Wäckers and Bonfay 2004). When plants produce toxic compounds for chemical defense, these compounds may be transferred to third and even fourth trophic levels, so that the effect of direct defence may reduce the effectiveness of indirect defence through negative effects of host quality on insect parasitoids (Harvey et al. 2003, Soler Gamborena et al. 2005). Such trade offs between direct and indirect defence, or between indirect defences, have been far less well studied below ground.

**Linking above and belowground multitrophic interactions**

Above and belowground multitrophic interactions may influence each other (Van der Putten et al. 2001), for example by belowground inductions of secondary plant compounds that become concentrated in the leaves (Van Dam et al. 2003). Currently, studies are being carried out that link below to aboveground interactions. For example, wireworms that feed on the roots of cotton change the spatial distribution of army worms on the leaves of the plants (Bezemer et al. 2003), which might influence attack of the army worms by their predators. Also, nematodes may reduce grass aphid populations, while the few aphids become bigger, which enhances aphid attack by parasitoids (Bezemer et al. 2005). It is also possible that belowground insects are reduced by the feeding of aboveground insects through reduced carbohydrate storage in the roots (Masters and Brown 1992).

Another interesting line of development is to include the role of biodiversity in the functioning of multitrophic interactions (Cardinale 1993). This concept has also been used in soils, for example in relation to pathogen control by compost. An interesting development here is to investigate how enemy control works in natural systems, which is the key issue of the EU-rtn project EcoTrain (2002-2006). Marram grass, a natural dune colonizer, is the
model system for that research project. Marram grass is attacked by a number of root-feeding nematode species and the dune soil contains a wealth of nematode enemies (Van der Putten 2003). It appears that removing some nematode species (cyst and root lesion nematodes) from the soil increases the damage to plants by root knot nematodes (Brinkman et al. 2005). This suggests that control of one nematode pest may lead to enhanced damage by other nematodes.

![Flow chart of concept-driven experimental research](image)

I conclude that further developments in biocontrol might be achieved by a more conceptual approach, for example by combining the concept of multitrophic interactions with that on biodiversity-ecosystem functioning relationships. Also, the developing of an above-belowground multitrophic interactions concept (Van der Putten et al. 2001) may enhance the integrated nature of biological control. This requires collaboration of above and belowground biocontrol researchers.

**Acknowledgements**

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**References**


Microbial diversity in wheat rhizosphere as affected by genetically modified \textit{Pseudomonas putida} WCS358r

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Abstract: Introduction of genetically modified micro-organisms (GMOs) in the environment can lead to perturbations of soil ecosystems. To monitor possible disturbances by GMOs, the impact of \textit{Pseudomonas putida} WCS358r and two genetically modified derivatives, which constitutively produce phenazine-1-carboxylic acid (PCA) or 2,4-diacetylphloroglucinol (DAPG), on the rhizosphere microflora of wheat was studied. The GMOs were introduced into soil as a wheat seed coating from 1999 till 2002. Every year the same treatment was applied in the same field plots. To compare a possible impact of the GMOs to that of common agricultural practice, a crop rotation of wheat and potato was incorporated in the field experiment and evaluated against continuous wheat cropping. The microflora was studied by extracting DNA directly from rhizosphere soil and subsequently amplifying rDNA using bacteria-specific primers. The resulting amplicons were separated using denaturing gradient gel electrophoresis (DGGE). In the first year all bacterial treatment had a transient impact on the indigenous bacterial microflora. After repeated introduction the DAPG-producing GMO had a significant effect on the bacterial microflora as from the second year, and the PCA-producing GMO after the fourth introduction. Cropping potato had a clear long-term impact that continued to exist in a subsequent wheat crop. The impact of the GMOs was never larger than that of changing crop from wheat to potato.

Rhizosphere samples of control wheat plants and of plants treated with wild-type bacteria or the GMOs were also analyzed using an Affymetrix GeneChip\textsuperscript{®} containing 16S rDNA sequences of approximately 9000 bacterial operational taxonomic units. Depending on the year and the treatment differences were observed in occurrence of specific bacterial groups.

Key words: 2,4-diacyetylphloroglucinol, denaturing gradient gel electrophoresis, genetically modified microorganisms, microarrays, microbial diversity, phenazine-1-carboxylic acid, 16S rDNA

Introduction

Large-scale introduction of genetically modified microorganisms (GMOs) in the environment might affect the natural soil ecosystem. In previous field experiments it was indeed demonstrated that introduction of a \textit{Pseudomonas putida} strain that was genetically improved in its biocontrol properties through the production of the antimicrobial compound phenazine-1-carboxylic acid (PCA), resulted in a transient shift in the natural soil microflora (Glandorf et al., 2001). Since under commercial cropping conditions GMOs will be introduced not only at a single time point but probably every year in the same field, the aim of the present field study was to determine whether repeated introduction of the GMOs in the same field intensifies the earlier observed shift in the microflora. Effects observed in the earlier studies may have been specific for PCA and, therefore, in the present study a second GMO was introduced that constitutively produced the antimicrobial compound 2,4-diacyetylphloro-
glucinol (DAPG) (Bakker et al., 2002; Viebahn et al., 2003, 2005). Since only a small part of the soil microflora can be cultured (Atlas and Bartha, 1998; Hugenholtz and Pace, 1996) cultivation-independent techniques were used to assess the effects of the introduced bacteria. DNA was isolated directly from rhizosphere samples, and small subunit (16S) ribosomal DNA (rDNA) was amplified with specific primers and subjected to denaturing gradient gel electrophoresis (DGGE) (Viebahn, 2005). DGGE can reveal changes in microbial communities, however, it is not suitable to identify the microorganisms that are affected by a certain perturbation. To identify bacterial species affected by the introduction of GMOs, additional molecular techniques were used. PCR amplified DNA of rhizosphere samples was hybridized to Affymetrix GeneChips® containing 16S rDNA sequences (DeSantis et al., 2003) of approximately 9000 operational taxonomic units (OTUs).

Material and methods

Bacterial strains and experimental field
For this field experiment the plant growth-promoting rhizobacterial strain P. putida WCS358r (Geels and Schippers, 1983) was modified with the phz biosynthetic gene locus of strain P. fluorescens 2-79 (Thomashow and Weller, 1988) that codes for the production of PCA. In addition, this strain was genetically modified with the phl biosynthetic gene cluster from P. fluorescens Q2-87 coding for the production of 2,4-diacetylphloroglucinol (DAPG) (Bangera and Thomashow, 1999; Viebahn et al., 2003). Both PCA and DAPG are antimicrobial compounds that have been implicated in suppression of plant pathogens by fluorescent pseudomonads. The genes were inserted into the chromosome of WCS358r by using a mini-transposon (Herrero et al., 1990). The constructs resulted in constitutive PCA or DAPG production by the GMOs (Glandorf et al., 2001; Viebahn et al., 2003). The GMOs were introduced into the soil as a coating on wheat seeds. To study possible synergistic effects of PCA and DAPG production, the GMOs were applied either separately or in combination. Every year the same treatments were applied in the same plots of the experimental field. Each treatment was repeated in 6 plots, and samples were collected each year at different time intervals after sowing (Viebahn et al., 2003). Effects of the GMOs on the natural microflora on plant roots were compared to effects of the parental strain. To compare effects caused by the GMOs with effects caused by common agricultural practice such as crop rotation, one treatment consisted of rotation plots, where in 1999 and 2001 wheat, and in 2000 and 2002 potatoes were grown.

DNA extraction and PCR
Three to five g of roots with adhering soil were sampled from each plot and mixed with 10 ml sodium phosphate buffer (120 mM, pH 8). One g of gravel was added and samples were vortexed for 30 s. The supernatant was decanted into a new tube. One ml of the supernatant was used to extract total DNA with the FastDNA®SPIN Kit for Soil (Bio 101, Biogene, Vista, Calif.) in combination with a Ribolysér (Hybaid, Ashford, UK), as previously described (Smit et al., 2003). The extracts were suspended 1:100 in 100 µl Millipore-filtered distilled water before purification with the Wizard® DNA Clean-up System (Promega, Madison, WI) according to the manufacturer’s protocol.

Amplification of the rDNA for the DGGE was done with the primer pair F-968-GC and 1491R (Nübel et al., 1996). F-968-GC contained a 40 bp GC-clamp to stabilize the melting behavior of the DNA fragments for DGGE analysis (Sheffield et al., 1989). The PCR was performed in 10x PCR buffer 2, pH 9.2 containing 2.25 mM MgCl₂ (Roche Diagnostics, Mannheim, Germany), 250 µM of each of the four deoxynucleoside triphosphates, 250 nM of each primer, 2.5 U Expand Long template enzyme (Roche, Diagnostics, Mannheim, Germany) and 1 µl of appropriately diluted template DNA. The PCR conditions used in the
thermocycler (Hybaid, Ashford, UK) were: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C, and finally 10 min at 72°C.

Amplification of rDNA for the microarray analysis were carried out using the primer pair 27F and 1492R (Lane, 1991), which amplify the 16S rRNA genes of a wide range of members of the domain Bacteria. The PCR was performed as described above, except that the concentration of each of the primers was 1 µM. The PCR conditions were: 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 53°C, and 1 min at 72°C, and finally 7 min at 72°C.

**DGGE**

PCR fragments were separated on a denaturing gradient polyacrylamide gel consisting with a urea formamide denaturant gradient of 30–60 %. A 100 % denaturing acrylamide gel contained 7 M urea and 40 % formamide. PCR products were loaded and gels were run for 17 h at 80 V at a constant temperature of 60°C in a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands). After electrophoresis gels were stained with SybrGold (Molecular Probes, Leiden, The Netherlands) and viewed under a blue light transilluminator (Clare Chemical Research, Dolores, Colorado). Images were digitalized using the GeneGenius Bio Imaging System (Syngene, Cambridge, UK). The bacterial community fingerprints of the DGGE gels were analyzed with the BioNumerics program vers. 3.5 (Applied Maths, Sint-Martens-Latem, Belgium).

**Microarray analysis**

Two µg DNA amplicons were prepared containing One-Phor-All Buffer Plus and 0.2 U DNAsae I per µg DNA (Invitrogen, Carlsbad, CA) in a 50 µl solution, and fragmentation was performed for 10 min at 25°C, followed by 10 min at 98°C. The fragmented products were biotin-labeled for 1 h at 37°C using the Enzo®BioArray Terminal Labeling Kit (Affymetrix, P/N 900181) according to the manufacturer’s protocol.

Hybridization was done according to the GeneChip® expression analysis technical manual as previously described (Masuda and Church, 2002). The arrays were scanned at 570 nm with a resolution of 3 µm with a GeneArray scanner (Affymetrix, Inc.). Data analysis was performed by using custom software, which scaled each array. Cluster analysis was performed with GeneMaths vers. 2.1 (Applied Maths, Sint-Martens-Latem, Belgium).

**Results and discussion**

**DGGE analysis**

For each year composite dendrograms representing three to four sampling dates were constructed. In 1999 all bacterial treatments clustered separately from the non-treated control plots (figure 1A, please note that the rotation plot (rp) was cropped to wheat in 1999). In the second year three clusters were apparent, one containing the samples from the plots cropped to potato, one with those treated with the DAPG-producing GMO, and one cluster containing the control treatment and samples treated with the wild type or the PCA producer (figure 1B). In 2001 again the crop rotation plots were different from the continuous wheat plots, whereas it was now cropped to wheat. All bacterial treatments affected the community composition; however, the DAPG producer had an effect differential from the wild type and the PCA-producing GMO (figure 1C). In 2002 the GMOs affected the bacterial community composition, their effects never exceeded those of changing crop from wheat to potato (figure 1D).

Introduction of a DAPG-producing derivative of *P. putida* WCS358r affected the bacterial microflora after repeated introductions. The PCA-producing GMO affected the bacterial microflora differentially from the parental strain only after 4 consecutive introductions. These effects never exceeded those of changing crops from wheat to potato.
Cropping potato once had a long lasting effect on the microflora, as it was still apparent one year later.

**Figure 1.** Composite dendrograms representing the genetic similarity of the bacterial communities of field-grown wheat plants in 1999 (A), 2000 (B), 2001 (C), and 2002 (D). Wheat seeds were treated with a control coating containing no bacteria (contr), the parental strain WCS358r (wt), the DAPG producer (phl), the PCA producer (phz) or the combination of both GMOs (phz+phl). In a rotation plot (rp) untreated wheat was sown in 1999 and 2001 and in 2000 and 2002 untreated potato was planted. The six replicates of each treatment were pooled to two replicate samples. Similarities are based on DGGE patterns generated from 16S rDNA fragments.

**Affymetrix GeneChip® data**

The GeneChip® data were used to construct dendrograms to identify similarity between treatments (data not shown). Two main clusters were apparent, one containing samples from the GMO-treated plots, and one from the control and wild-type treated plots. The same samples were subjected to DGGE, resulting in a similar dendrogram (figure 2). Analysis of the individual signals from the GeneChip® data revealed that bacterial diversity in the rhizosphere of control plants was lower than that in the rhizosphere of treatments with the bacteria, either wild type or GMO.
Introduction of antagonistic bacteria seemed to increase rather than decrease bacterial biodiversity in the wheat rhizosphere.

Figure 2. Dendrogram based on the genetic similarity of the bacterial communities of field-grown wheat plants in 2002 with the corresponding DGGE gel. Wheat seeds were treated with \textit{P. putida} WCS358r (wt), the DAPG-producing derivative of WCS358 (phl), the PCA producer (phz), or a control coating containing no bacteria (contr). Significant clusters are indicated by the gray line.

Acknowledgements

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References


Disease suppressive soils

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Abstract: Suppressive soils have been described worldwide for a broad spectrum of plant pathogenic fungi, bacteria and nematodes. Classical approaches employed to identify the microorganisms and mechanisms involved in suppression have changed little during the last four decades. However, when classical approaches are used in combination with sophisticated techniques of molecular ecology, it is possible to more rapidly define and characterize the agents and mechanisms responsible for specific suppression. Mechanisms of suppressiveness operating in several suppressive soils are discussed below.

Key words: Pseudomonas, Fusarium, Streptomyces, Heterodera, apple replant disease, biological control, soilborne pathogens, take-all decline

Introduction

Suppressive soils were defined by Baker and Cook (1974) as “soils in which the pathogen does not establish or persist, establishes but causes little damage, or establishes and causes disease for awhile but thereafter the disease is less important although the pathogen may persist in the soil.” Conducive (nonsuppressive) soils are those in which disease readily occurs. Suppressive soils have been described worldwide for a broad spectrum of plant pathogenic fungi, bacteria and nematodes (Weller et al., 2002). Throughout history, farmers have unknowingly used suppressive soils to control soilborne pathogens, recognizing only that a crop grew better in one field than in others. Only in the last 40 years have researchers unraveled the basis of suppressiveness in some soils. An excellent “snapshot” of the importance of soil suppressiveness in agriculture during the 20th century was captured in the book “Ecology of Soil-Borne Plant Pathogens - Prelude to Biological Control” (Baker and Snyder, 1965), which recorded the proceedings of an International Symposium on Factors Determining the Behavior of Plant Pathogens in Soil, Berkeley, CA, 1963. The following comments about monoculture and soil suppressiveness were recorded (pp. 434-435) during a discussion of M.D. Glynne’s paper, “Crop Sequence in Relation to Soil-Borne Pathogens.” For example S. D. Garrett said, “both eyespot and take-all diseases of cereals at first increase with continuous cultivation of a susceptible crop, but after a few years significantly decline. This also happens with Phymatotrichum root rot of cotton.” J.E. DeVay added, “corn is commonly cropped on the same fields in Minnesota; … no increase in diseases such as root- and stalk-rot is apparent. Yields of corn from fields continuously cropped to corn are comparable to those from similar stalk populations on fields involved in various crop rotations.” J. W. Oswald then said, “I would like to add to the list of diseases showing this phenomenon. We have potato rotation plots now that are in their 14th year which showed a buildup of potato scab to a maximum in about the fifth year, and then a gradual declined.” E. K. Vaughan then said, “In western Oregon they (onions) have been grown continuously in many fields for at least 65 years in pure organic soil (deep peat), of very low pH, which remains wet and cool most of the year. Under these conditions pink rot, Pyrenochaeta terrestris, is always present, yet good yields are obtained.”
In this paper, the properties and characteristics of suppressive soils, the strategies used to determine the microbial basis of suppressiveness, and the mechanisms(s) of disease suppression operating in several suppressive soils are briefly discussed.

General and specific suppression

Suppressive soils owe their activity to a combination of general and specific suppression. General suppression: i) is a characteristic of essentially all natural soils to inhibit the growth of soilborne pathogens to a limited extent; ii) results from the activity of the total microbial biomass in soil; iii) is often increased by the addition of organic matter, the buildup of soil fertility and other practices that enhance the microbial activity of the soil; iv) is not transferable between soils; and v) usually survives when the soil is heated to 70°C, and partially survives soil fumigation with methyl bromide (Cook and Rovira, 1976; Weller et al., 2002).

Specific suppression: i) is superimposed over the background of general suppression; ii) is much more highly effective than general suppression; iii) results from individual or select groups of microbial species; iv) is transferable to a conducive soil in the greenhouse or field; and v) often is eliminated by pasteurizing (55-60°C for 30 min.), fumigating or gamma irradiating the soil (Cook and Rovira, 1976; Gerlagh, 1968; Weller et al., 2002).

Specific suppression has also been defined on the basis of the longevity of the suppressiveness. Long-standing suppressiveness is naturally associated with the soil, its origins are not known and it persists in the absence of plants. In contrast, induced suppressiveness is initiated and sustained by practices such as crop monoculture and continuously growing crops susceptible to the target pathogen (Hornby, 1983, 1998; Weller et al., 2002).

Determining the microbial basis of specific suppression

Thousands of bacterial and fungal species and subspecies inhabit a gram of soil, making the identification of individual microorganisms or select groups of organisms responsible for specific suppressiveness in a given soil a very challenging process. The “classical” approaches or strategies most commonly employed to identify the microorganisms involved in suppression have changed little during the last four decades and include the following (Weller et al., 2002).

1. Transfer suppressiveness by adding 0.1-10% suppressive soil to conducive soil to confirm its biological nature.
2. Treat the soil with heat, biocides or antibiotics to eliminate specific microbial groups and then assay for loss of suppressiveness.
3. Isolate different microbial groups and correlate their presence with suppressiveness.
4. Screen representative strains of microbial groups for biocontrol activity against the target pathogen.
5. Convert conducive soil into suppressive soil by introducing representative strains.
6. Phenotypically characterize the microorganisms(s) conferring suppressiveness.
7. Elucidate the mechanism(s) of suppressiveness of the microbial agent(s).

A variety of more sophisticated techniques developed over the last 15 years are now being applied to the study of suppressive soils, and when used in combination with classical techniques have significant potential to more rapidly define and characterize the agents and mechanisms responsible for specific suppression. The following list of “contemporary” approaches or strategies available to study suppressive soils was adapted from the reviews of Mazzola (2004) and Garbeva et al., (2004), which describe the strengths and weakness of each technique.
8. Use of probes and primers to genetic targets in suspect suppressive agents, along with colony hybridization, PCR (Raaijmakers et al., 1997; de Souza et al., 2002 and/or real-time PCR (Vandemark et al., 2002) to monitor the population dynamics of agents and target pathogens in suppressive and conducive soils.

9. Analyze microbial community composition of suppressive and conducive soils by:
   a) community-level physiological profiling, for example with the BIOLOG® system (Campbell et al., 1997; Lupwayi et al., 1998).
   b) phospholipid fatty acid (PLFA) or fatty acid methyl ester (FAME) analysis (Ibekwe and Kennedy, 1999; Larkin, 2003).
   c) analysis of ribosomal RNA genes (rDNA) by: denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) (Muyzer and Smalla, 1998); terminal restriction fragment length polymorphisms (R-RFLP) (Liu et al., 1997; McSpadden Gardener and Weller, 2001); oligonucleotide fingerprinting of ribosomal RNA genes (Yin et al., 2003a,b).

10. Analyze genomic libraries derived from soil (metagenomic libraries) (Sebat et al., 2003)

Examples of specific suppressiveness

Suppression of bacterial pathogens
Common scab is a chronic disease of potato caused by *Streptomyces scabies* and other *Streptomyces* species (Conn et al., 1998). In the mid-20th century, the decline of potato scab was observed in fields that had undergone many years of potato production and potato monoculture (Lorang et al., 1989; Menzies, 1959). Suppressiveness is transferable (1-10% suppressive soil) under both field and controlled conditions and eliminated by heating the soil (Bowers et al., 1996; Menzies, 1959). Nonpathogenic strains of *Streptomyces* from disease-free potatoes grown in suppressive soil were inhibitory to pathogenic *S. scabies* in vitro through antibiotic production (Liu et al., 1996; Lorang et al., 1989; Lorang et al., 1995). Addition of scab-suppressive isolates *S. diastatochromogenes* and weakly pathogenic *S. scabies* into conducive soil infested with the pathogen resulted in scab suppression that persisted in the soil (Bower et al., 1996; Liu et al., 1995, 1996; Ryan and Kinkel, 1997). Suppressive *Streptomyces* isolates with the greatest antibiotic production and most vigorous growth suppressed the disease better than isolates showing less antibiosis and aggressive growth (Liu et al. 1996). Although the basis of potato scab decline has not been fully elucidated, antagonistic *Streptomyces* are key facilitator of suppressiveness by competing with the pathogen for resources and direct inhibition by antibiotics (Weller et al., 2002).

Suppression of nematodes
Soils suppressive to cyst nematodes are well known and in them densities of cyst nematodes typically remain low in the presence of a susceptible host. Fungi parasitizing eggs, females and cysts often are associated with the decline of cyst nematodes in soils (Kerry, 1988; Westphal and Becker, 2001). One of the best examples of nematode suppression is the decline of *Heterodera avenae* (Kerry et al., 1980), resulting from the parasitic activity of *Nematophthora gynophila* and *Verticillium chlamydosporium*.

Field 9E at the agricultural research station of the University of California, Riverside became suppressive to *Heterodera schachtii* after the continuous cropping of susceptible crops (Westphal and Becker, 1999). Suppression was apparent by the occurrence of lower nematode densities and multiplication in the suppressive compared to the conducive soil. Suppressiveness was eliminate by soil treatment with metam sodium, methyl bromide, methyl iodide, formaldehyde and heat (>55°C, 30 min.) (Westphal and Becker, 1999). The suppressiveness was transferable by adding 1.0 to 10% suppressive soil or cysts that developed in suppressive soil into conducive soil (Westphal and Becker, 2000; 2001). The
most common fungi isolated from cysts were *Fusarium oxysporum*, *Fusarium* sp. nov. and *Dactylella oviparasitica* (Westphal and Becker, 2001). Fungi associated with cysts from soils possessing different levels of suppressiveness were then examined by the nonculture-based analysis, oligonucleotide fingerprinting of ribosomal RNA genes (OFRG). Only *D. oviparasitica*-like DNA was consistently associated with cysts from highly suppressive soil, indicating that this fungus was involved in *H. schachtii* suppression (Yin et al., 2003). This work is perhaps the best example to date of the use of classical and contemporary approaches in combination to identify the agent(s) responsible for specific suppression.

A field located at the University of Florida, Green Acres Agronomy Research Farm near Gainesville, Florida was sown continuously to tobacco for seven years without the application of nematicides or other nematode management practices (Chen et al., 1994). Over the years of monoculture, root-knot damage caused by *M. incognita* race 1 and *M. javanica* declined until there was little difference in the growth of nematode susceptible and resistant tobacco cultivars in the soil. The decline in disease was related to the occurrence of endospores of the obligate parasite *Pasteuria penetrans* in mature females and attached to second-stage juveniles (J2) of both *M. incognita* and *M. javanica* collected from the field (Weibelzahl-Fulton, et al., 1996). This suppressive soil is a good example of an exception to the general observation that most specific suppressiveness is eliminated by soil pasteurization; endospores of *P. penetrans* are resistant to high temperature and require autoclaving to eliminate them from the soil (Weller, et al., 2002).

**Suppression of soilborne fungal pathogens**

The best example of long-standing suppressiveness is found in soils suppressive to Fusarium wilts, caused by pathogenic *F. oxysporum* (Weller et al., 2002). Studies of the Chateaucrenard (France) (Alabouvette, 1986; Alabouvette et al., 1993) and Salinas Valley (California, USA) (Scher and Baker, 1980) soils provided the foundation for the current knowledge about agents(s) and mechanisms responsible for wilt suppression. Also notable are soils in Florida, USA suppressive to *F. oxysporum* f. sp. *niveum*, which are not long-standing but induced by continuous cropping of partially resistant watermelon cultivars (Hopkins et al., 1987; Larkin et al., 1993). In general wilt suppressiveness is eliminated by treatment with moist heat (>55 C), methyl bromide and gamma radiation, and transferred by mixing 1-10% suppressive soil into conducive soil (Alabouvette, 1986; Scher and Baker, 1980). Suppressiveness results primarily from the activity of nonpathogenic *F. oxysporum*, with fluorescent pseudomonads (in some cases) creating a more effective suppression. In general, mechanisms of both agents include competition and induced resistance (Alabouvette, 1990; Duijff et al., 1998; Duijff et al., 1999; Lemanceau et al., 1993).

In Washington State, USA, apple replant disease, the poor growth of apple trees in orchard soil previously cropped to apple, is caused by a complex of soilborne fungi including *Rhizoctonia solani* (Mazzola, 1998). Mazzola (Mazzola, 1999, 2004) demonstrated that: i) wheat field or virgin soils were suppressive to disease on apple seedlings caused by *R. solani* AG-5; ii) soil from adjacent ground in apple production was conducive to disease; iii) orchard soils became progressive more conducive to disease the longer the orchard was in production; iv) a reduction in the suppressiveness of a soil was associated with a precipitous decline in densities of *Pseudomonas putida* and *Burkholderia cepacia*, known antagonists of *R. solani* and other soilborne pathogens; v) introduction of *P putida* from wheat soil enhanced the growth of apple seedlings in replant soil (Mazzola, 1998; Weller et al., 2002); and vi) cultivation of orchard soil with wheat prior to planting apple seedlings increased rhizosphere populations of *P. putida* and suppressiveness of the soil (Mazzola and Gu, 2000a,b; Weller et al., 2002). Although the exact agent(s) responsible for disease suppression has not been conclusively identified, GC-FAME analysis indicated a clear relationship between the composition of the fluorescent pseudomonad community and suppressiveness (Mazzola, 2004).
This suppressive soil system is one of the most elegant for conducting fine-scale studies of changes in microbial community structure associated with a shift in the soil from a suppressive to a conducive state. This is because of the availability of field soils showing a gradient of activity ranging from highly suppressive (wheat field soil) to highly conducive (long-term apple orchard soil), and wheat cultivars differing in ability to modify the fluorescent pseudomonad community structure and convert and apple replant conducive soil into a suppressive soil (Mazzola and Gu, 2000a,b; Weller et al., 2002).

Take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici*, remains among the most important root diseases of wheat worldwide, despite being one of the most-studied. Take-all decline (TAD), the best example of induced suppression, is defined as the spontaneous decrease in the incidence and severity of take-all that occurs with monoculture of wheat or other susceptible host crops after a severe outbreak of the disease (Weller et al., 2002). TAD is a field phenomenon that occurs worldwide (Gerlagh, 1968; Hornby, 1998; Weller et al., 2002) and its development follows a consistent pattern, with environmental factors and previous history only modulating the speed of its onset. The suppressiveness associated with TAD is transferable to conducive soils in the greenhouse and the field, is eliminated by soil pasteurization (60°C, 30 min.) or fumigation and is reduced or eliminated by breaking monoculture with a non-susceptible crop (Cook and Rovira, 1976; Gerlagh, 1968; Raaijmakers and Weller, 1998).

Microbial changes in the bulk soil and/or rhizosphere resulting in antagonism of *G. graminis* var. *tritici* are the mechanisms most commonly reported to be responsible for TAD (Hornby, 1998; Weller et al., 2002). Several studies have provided compelling microbiological and biochemical evidence that the buildup of fluorescent *Pseudomonas* spp. producing the broad-spectrum, polyketide antibiotic 2,4-diacytethylphloroglucinol (2,4-DAPG) during wheat monoculture plays a key role in the suppressiveness of TAD soils (Weller et al., 2002) in both Washington State, USA (Raaijmakers et al., 1997, 1999; Raaijmakers and Weller, 1998) and in The Netherlands (de Souza et al., 2002). Take-all suppression occurs when populations of 2,4-DAPG producers reach a threshold density of $10^5$ CFU/g of root (Raaijmakers and Weller, 1998). The antibiotic is produced in the rhizosphere and easily isolated (Raaijmakers et al., 1999; Bergsma-Vlami et al., 2005), and *G. graminis* var. *tritici* is highly sensitive to it.

Evidence is mounting that 2,4-DAPG producers play a much broader role in plant defense against soilborne pathogens during crop monoculture. For example, Landa et al (2002) reported that densities of 2,4-DAPG producers were $>10^5$ CFU/g root on wheat or pea grown in soil from a field in Mt. Vernon, WA that had undergone over 30 years of pea monoculture and is suppressive to Fusarium wilt of pea and take-all (J.M. Raaijmakers and D.M. Weller, unpublished data). On the campus of North Dakota State University, Fargo, three side-by-side field plots occur where for over 100 years wheat or flax has been sown continuously or crop rotation practiced. Population densities of 2,4-DAPG producers were $>10^5$ CFU/g of root on wheat or flax sown in the monoculture soils but were barely detectable on roots grown in the crop rotation soil. Exactly how and why 2,4-DAPG producers are enriched during crop monoculture remains unknown but is a focus of current research.

All strains of 2,4-DAPG-fluorescent pseudomonads identified to date are *P. fluorescens*, however genotyping based on genomic fingerprinting by RAPD analysis (Keel et al., 1996; Mavrodi et al., 2001; Picard et al. 2000; Raaijmakers and Weller, 2001), whole-cell repetitive sequence-based (rep)-PCR analysis (Landa et al., 2002; McSpadden Gardener et al., 2000) and RFLP analysis of *phlD* (a key gene in the 2,4-DAPG biosynthetic locus) (Landa et al., 2002; McSpadden Gardener et al., 2001; Ramette et al., 2001; Wang et al., 2001) has revealed a considerable amount of variation among 2,4-DAPG producers (Weller et al.,
The clusters defined by the different genomic fingerprinting methods or by sequence analysis of *phlD* correlate well. Currently, twenty two genotypes (designated A to T and PfY and PfZ) have been defined by rep-PCR with the BOXA1R primer (BOX-PCR) or RFLP analysis of *phlD* (Landa et al., 2002; Mazzola et al., 2004; McSpadden Gardener et al., 2000, 2005), and the genotype is highly predictive of the ability of an isolate to colonize the rhizosphere of wheat (Landa et al., 2003) and pea. For example, D-genotype isolates, the most abundant genotype in Washington TAD soils (Raaijmakers and Weller, 2001), aggressively colonize wheat roots when applied at very low doses, thus accounting for their role in TAD in Washington State.

**Conclusions**

Throughout the centuries, suppressive soils have been important forces in the control of soilborne pathogens without farmers even knowing of their existence. Today, they continue to be and important but underutilized resource for controlling diseases with minimal application of off-farm inputs, and should be considered key management tools in sustainable agriculture. They remain excellent models for understanding the processes and mechanisms of biological control, and serve as rich sources of new biocontrol agents. By coupling the sophisticated techniques of molecular ecology with classical approaches for studying suppressive soil, there is tremendous potential to more rapidly define and characterize the agents and mechanisms responsible for specific suppressiveness.

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**References**


Effects of beneficial microorganisms on plants

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Abstract: Non-pathogenic soilborne microorganisms can promote plant growth, as well as suppress diseases. Plant growth promotion is taken to result from improved nutrient acquisition or hormonal stimulation. Disease suppression can occur through microbial antagonism or induction of resistance in the plant. Several rhizobacterial strains have been shown to act as plant growth-promoting bacteria through both stimulation of growth and induction of systemic resistance (ISR), but it is not clear in how far both mechanisms are connected. ISR results from the specific recognition of (a) bacterial determinant(s) by (a) plant receptor(s) on the roots and is manifested as a reduction of the number of diseased plants or in disease severity upon subsequent infection by a pathogen. ISR is effective against both soilborne and foliar pathogens and its demonstration requires spatial separation of the inducing bacterium and the challenging pathogen. The spectrum of diseases to which ISR confers enhanced resistance overlaps partly with that of systemic acquired resistance (SAR), which is induced as a result of limited infection by pathogenic microorganisms. Both ISR and SAR represent a state of enhanced basal resistance of the plant that depends on the signaling compounds jasmonic acid and salicylic acid, respectively, and for which sensitivity to ethylene is required. Pathogens are differentially resisted by these resistance signaling pathways, which can explain the differential effectiveness of ISR and SAR against biotrophic and necrotrophic pathogens, as well as their additive effects against pathogens that activate both resistance signaling pathways.

Key words: biological control, growth promotion, induced systemic resistance, rhizobacteria, systemic acquired resistance

Introduction

Soilborne diseases are difficult to control. Physical methods such as steaming of the soil are costly, and solarization and inundation are effective only in (sub)tropical climates. While such treatments are highly effective in reducing the number of propagules of pathogenic microorganisms and nematodes, they leave the soil vulnerable to rapid re-establishment of pathogens due to the elimination of antagonistic fungi and bacteria. Chemical methods are environmentally undesirable because large amounts of toxic compounds have to be used and pathogenic microorganisms tend to develop tolerance. Breeding for resistance is often not possible because of a lack of sources of effective resistance genes.

Antagonistic microorganisms in soil can suppress diseases as well as promote plant growth. Inoculation of soils or treatment of seeds with such microorganisms leads to better plant stand and increased crop yields. Particularly rhizosphere-colonizing bacteria can have beneficial effects and are often referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1980). Many rhizobacterial isolates have been shown to reduce diseases by antagonizing soilborne pathogenic fungi and bacteria through competition for nutrients, particularly iron, production of antibiotics, or secretion of lytic enzymes (Handelsman and Stabb, 1996). However, beneficial rhizobacteria can also act through effects on the plant: they can promote plant growth and enhance plant defensive capacity through induction of systemic resistance (Van Loon and Bakker, 2003). Both activities may be linked, as appears to apply to the effects of *Bacillus subtilis* strain GB03 and *Bacillus amyloliquefaciens* strain IN937a on *Arabidopsis*, where the bacterially produced volatile 2,3-butanediol has been demonstrated...
both to promote plant growth and to induce resistance against the bacterial pathogen *Erwinia carotovora* pv. *carotovora* (Ryu et al., 2003b, 2004b). However, the mechanisms involved are not known. An interpretation that cannot be easily discounted is that the increased vigour of bacterized seedlings makes them more resistant to pathogens because plants outgrow the sensitive seedling stage more quickly. This pertains particularly to infections by soilborne fungi that cause damping-off, such as *Pythium* spp., *Fusarium oxysporum* and *Rhizoctonia solani*. There are many reports describing enhanced growth and reduced disease development in soils containing these pathogenic fungi upon treatment of the soil or seeds with specific rhizobacterial strains (Van Loon and Bakker, 2005b), but it is difficult to interpret these findings in terms of microbial antagonism, growth promotion and/or induction of resistance.

**Promotion of plant growth**

Part of the uncertainty about mechanisms involved derives from the aim of many studies to identify rhizobacterial strains that are effective in increasing crop yields. Typically, to this end bacteria are isolated from the rhizosphere of the plant species concerned, usually tested for antagonism against selected pathogens in vitro on artificial media, and subjected to bioassays in inoculated or natural soils. Antibiotic production in vitro is not predictive of effectiveness in vivo, meaning that under the highly competitive conditions prevailing in the rhizosphere of plants bacterial metabolism can be very different from that on laboratory media (Haas and Keel, 2003). Therefore, screening for effectiveness can only be done reliably by performing bioassays under carefully controlled conditions. If improved plant performance is found as a result of root bacterization, it is desirable to analyze the mechanisms involved. The ease of generating bacterial mutants that are impaired in specific traits has made it possible to define the processes involved and to identify bacterial determinants that are responsible for the growth-stimulating effects observed.

As a representative example of improved plant performance, coating radish seeds with the rhizobacterial strain *Pseudomonas fluorescens* WCS374 improved plant stand in a commercial greenhouse with soil containing the vascular wilt fungus *Fusarium oxysporum* f.sp. *raphani*, reduced disease by about 50%, and increased tuber yield by 30-50% (Leeman et al., 1995c; R.J. Scheffer, personal communication). Whether growth promotion by the bacteria could be the mechanism involved was investigated in experiments in which radish was grown in vitro under gnotobiotic conditions. The presence of WCS374 bacteria increased leaf dry weight by 36%, indicating that WCS374 can behave as a PGPR. In autoclaved soil, another *P. fluorescens* strain, WCS417r, increased fresh weight of *Arabidopsis* plants by 32% (Pieterse and Van Loon, 1999), indicating that growth promotion is a fairly common property of *P. fluorescens* strains. Under gnotobiotic conditions, WCS417r increased root length of *Arabidopsis* seedlings growing vertically on an agar medium containing half-strength Hoagland solution (M.H. Thijssen, unpublished observation). Proliferation of lateral roots was also observed (S. van der Ent, unpublished observation). These effects resemble those of an application treatment with the plant hormone auxin.

Many bacteria have the ability to produce auxins, as well as growth-promoting gibberellins and cytokinins and growth-inhibitory ethylene (Frankenberger and Arshad, 1995). It has often been inferred that rhizobacterially produced auxins are responsible for growth promotion. However, compared with shoot elongation, root growth is only slightly stimulated by auxin, and generally only at concentrations of $10^{-9}$ to $10^{-11}$ M, higher concentrations being strongly inhibitory (Thimann, 1937). If the auxin concentration reached in the root after uptake of bacterially produced indoleacetic acid (IAA) does not fall within the limits indicated above, no root growth promotion can be expected. Auxin is transported basipetally towards the root tip, but some might be re-transported to the shoot. However,
concentrations required for shoot growth are unlikely to be reached. On the other hand, auxin at $10^{-4}$ to $10^{-6}$ M promotes lateral root formation, and it cannot be excluded that locally bacterial microcolonies can produce auxin in amounts that could stimulate this process and, thereby, contribute to enhanced uptake of water and nutrients. For example, mutant strains of the PGPR *Azospirillum brasilense* that synthesized very low amounts of IAA compared to the wild-type strain, no longer promoted the formation of lateral roots of wheat seedlings (Barbieri et al., 1986; Barbieri and Galli, 1993).

The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is exuded by growing plant roots together with other amino acids, organic acids and sugars, and metabolized by bacterial strains that hydrolyze ACC by deamination to ammonia and α-ketobutyrate. By reducing the level of exuded ACC, re-uptake by the plant would be lowered, less ethylene would be produced and, consequently, roots grow longer (Glick et al., 1994, 1998). The ability to utilize ACC as a sole nitrogen source appears to be limited to soil bacteria that are capable of stimulating plant growth (Glick et al., 1995), linking ACC-deaminating activity to growth promotion. When the PGPR *Pseudomonas putida* strain GR12-2 was mutagenized to select for variants that were unable to utilize ACC, those mutants proved to be devoid of the ACC-deaminase activity that is present in wild-type GR12-2 cells. They had also lost the ability to promote root elongation of developing canola (*Brassica campestris*) seedlings under gnotobiotic conditions (Glick et al., 1994), and no longer promoted shoot growth of seedlings planted in soil (Glick et al., 1997).

“Mycorrhization helper bacteria” have been demonstrated to promote mycorrhizal development, notably of forest trees (Garbaye, 1994). Other suggested mechanisms of growth promotion comprise nitrogen fixation and stimulation of ion uptake. Some PGPR such as *A. brasilense* possess the ability to fix nitrogen (Steenhoudt and Vanderleyden, 2000), but its significance in the rhizosphere has not been critically assessed. Stimulation of ion uptake involves mainly solubilization of iron and phosphate from soils. Plants can benefit from the ferric-chelating properties of bacterial siderophores. Pseudobactin 358, the pyoverdin siderophore of *P. putida* strain WCS358, increased iron uptake and stimulated chlorophyll synthesis in barley (Duijff et al., 1994). Phosphate can be made available through the release of organic acids or unidentified enzymatic activities (Illmer and Schinner, 1992). Combined inoculation of *A. brasilense* and the phosphate-solubilizing bacteria *Pseudomonas striata* or *Bacillus polymixa* significantly increased nitrogen and phosphorus content as well as grain yield of sorghum (Alagawadi and Gaur, 1992). Which mechanisms are responsible for the growth promotion in radish and *Arabidopsis* by *P. fluorescens* strains WCS374 and WCS417, respectively, has not been investigated.

**Induction of systemic resistance**

To test whether induction of systemic resistance also played a role in the observed growth promotion of radish, bioassays were conducted in which the bacterial strain WCS374 and the pathogen, *F. oxysporum* f.sp. *raphani*, were inoculated at separate sites on the main root of radish seedlings (Leeman et al., 1995a). Because the bacteria and the fungus never contacted each other, a reduction in the percentage of plants developing wilting and internal browning symptoms was the result of a plant-mediated effect. This induction of resistance in the plant exhibited the typical characteristics of systemically induced resistance (Van Loon et al., 1998): through the microbial stimulation the defensive capacity of the plant was enhanced, the enhanced defensive capacity was expressed systemically, the induced resistance was active against fungi and bacteria, and once induced, the systemic resistance was maintained for prolonged periods. In many published papers claiming involvement of induced resistance in disease suppression and increased crop yield, the criterion of spatial separation between the rhizobacteria and the
pathogen is not met (Van Loon and Bakker, 2005b). Therefore, when interpreting these studies it is not possible to exclude microbial antagonism, particularly when protection is observed against soilborne pathogens. Disease suppression through induction of resistance against foliar pathogens by bacteria on the roots is more likely, but some rhizobacterial strains have the capacity to colonize the shoot also (Yan et al., 2003). Absence of the inducing rhizobacteria on the leaves must therefore be demonstrated.

Some rhizobacterial strains can induce systemic resistance in several plant species, whereas others appear to be more restricted (Van Loon and Bakker, 2005a,b). Thus, *P. fluorescens* WCS417 induces resistance in both radish and *Arabidopsis*, whereas WCS374 does so only in radish. In contrast, *P. putida* strain WCS358, which induces systemic resistance in *Arabidopsis*, does not do so in radish (Leeman et al., 1995a, Van Wees et al., 1997). Of the two strains that induce systemic resistance in radish, WCS374 and WCS417, killed cells were as effective as live ones. Thus, the resistance-inducing action of these strains could not be ascribed to active metabolites of the bacteria. Bacterial mutants that lacked the O-antigenic side-chain of the outer membrane lipopolysaccharide (LPS) no longer induced resistance, whether live or dead, indicating that the LPS acts as the inducing determinant of the systemic resistance in radish. In soil an LPS’ mutant of WCS374 failed to improve growth and suppress Fusarium wilt, demonstrating that the growth promotion and disease suppression observed could be explained fully by the induction of systemic resistance by this strain (Leeman et al., 1995b). Hence, although WCS374 can also promote growth under gnotobiotic conditions, this mechanism did not appear to be involved in the yield increase of soil-grown plants, and neither did microbial antagonism in the rhizosphere seem to play a major role.

The WCS374-induced systemic resistance resembled pathogen-induced systemic acquired resistance (SAR), the phenomenon that once a plant has been infected by a pathogen and been able to withstand it, it acquires an enhanced defensive capacity against subsequent infection by the same and other pathogens (Van Loon, 2000). SAR depends on a signaling pathway involving the hormonal compound salicylic acid (SA), as well as the regulatory protein NPR1, and is associated with the activation of genes encoding pathogenesis-related proteins (PRs) with potential antimicrobial activities. By using *Arabidopsis* as a model plant, *P. fluorescens* WCS417 as the inducing rhizobacterium, and the causal agent of bacterial speck disease, *Pseudomonas syringae pv. tomato* (Pst) as the challenging pathogen, it was found that induction of SAR by pretreatment with avirulent Pst or induction of systemic resistance (ISR) by root colonization with WCS417 reduced both the number of leaves developing symptoms and the proliferation of the virulent pathogen in the leaves to similar extents. However, whereas SAR was associated with the induction of mRNAs coding for the characteristic *Arabidopsis* PRs -1, -2 and -5, no such induction was apparent in the case of ISR (Pieterse et al., 1996). Transgenic NahG plants containing a gene from *P. putida* that hydroxylates SA, resulting in the formation of non-SAR-inducing catechol, were unable to express SA-dependent SAR, but unimpaired in the expression of ISR. Conversely, *Arabidopsis* mutants deficient in the perception of the hormones jasmonic acid (JA) or ethylene were protected through SAR to the same extent as wild-type plants, but WCS417 no longer elicited ISR in these mutants (Pieterse et al., 1998). These results demonstrated that SAR and ISR depend on different signaling pathways, requiring elevated levels of SA, and JA as well as ethylene perception, respectively. However, when mutant npr1 plants were tested, both SAR and ISR were abolished. Thus, both SAR and ISR require NPR1, in spite of the fact that downstream of NPR1, PRs are induced in SAR but not in ISR.

Both types of induced resistance also differ in the effectiveness of protection against different pathogens. NahG *Arabidopsis* plants are more susceptible to biotrophic and hemibiotrophic pathogens, indicating that SA is required for basal resistance against these types of pathogens. SA-dependent SAR is most effective against these same pathogens. In contrast, *Arabidopsis* mutants that are deficient in JA or ethylene perception are more susceptible to
necrotrophic pathogens, as well as to hemi-biotrophs, and ISR is most effective against these types of pathogens. The biotrophic downy mildew oomycete *Peronospora parasitica* was resisted through SAR and only weakly through ISR, whereas turnip crinkle virus was not resisted through ISR at all. In contrast, the necrotrophic leaf-spotting fungus *Alternaria brassicicola* was resisted solely through ISR and not at all through SAR. Pathogens with mixed lifestyles, such as the bacterial pathogens *Pst* and *Xanthomonas campestris* pv. *armoraciae*, were resisted substantially through both SAR and ISR (Ton et al., 2002). These differential effectiveness are marked by an enhanced expression of SA-responsive defense-related genes, such as *PR-1*, *-2*, and *-5*, upon challenge inoculation of SAR-expressing plants, and stimulated expression of JA-responsive defense-related genes, such as *Vsp*, when ISR-expressing plants are challenged (Van Wees et al., 1999). The effectiveness of both SAR and ISR against pathogens that are neither exclusively biotrophic nor entirely necrotrophic made it possible to increase protection by combination of ISR elicitation by rhizobacteria and SAR induction by an avirulent pathogen. In doubly induced plants protections as a result of ISR and SAR were additive against *Pst* and *X. campestris* pv. *armoraciae*, but not higher against *A. brassicicola* or turnip crinkle virus, against which only ISR and SAR, respectively, are effective (Van Wees et al., 2000; J.A. van Pelt and C.M.J. Pieterse, unpublished observations).

In the literature, specific resistance-inducing rhizobacterial species have been described to induce SA-dependent *PR* gene expression or PR-protein accumulation (e.g. Park and Kloepper, 2000; Coventry and Dubery, 2001). These observations suggest that those strains activate the SAR rather than the ISR signaling pathway, either by producing SA by themselves, or by activating the plant to produce SA. Such SA-dependent resistance is abolished in SA-degrading NahG plants. NahG transformants are available for *Arabidopsis*, tobacco and tomato, and have been tested for induction of systemic resistance by several rhizobacterial strains against a number of pathogens (Table 1). In almost all cases, ISR was maintained in the NahG plants, indicating that SA did not play a role in the rhizobacterially-induced resistance. Only in the case of *Pseudomonas aeruginosa* strain 7NSK2 on tobacco and tomato – but not on *Arabidopsis* – ISR was lost. On tomato, 7NSK2-induced resistance was shown to be elicited through the production by the bacteria of a combination of the siderophore pyochelin and the antibiotic pyocyanin (Audenaert et al., 2002). Pyochelin contains a SA-moiety. Hence, the requirement for SA pertains to the production of pyochelin, rather than to an inductive action of the compound itself. However, in tobacco, it was shown that SA production by the plant was also required (De Meyer et al., 1999). Notably, induction of ISR by 7NSK2 in *Arabidopsis* did not require SA (Ran et al., 2005). Thus, the interaction and ISR-eliciting characteristics of rhizobacterial strains differ for different plant species, as is also evident from the differential induction of ISR by strains WCS358, WCS374 and WCS417 in *Arabidopsis* and radish. Apparently, different bacterial determinants are perceived by different plant species. Depending on the strain and the host plant, LPS, siderophores and other iron-regulated compounds, antibiotics, flagella and volatile alcohols have been demonstrated to possess ISR-eliciting activity (Van Loon and Bakker, 2005a). Some strains can trigger ISR by more than a single factor, such as WCS374 in radish. LPS is the main inducing factor, but under iron-limiting conditions the pseudobactin siderophore and an additional iron-regulated compound can also elicit ISR (Leeman et al., 1996). In *Arabidopsis*, WCS358 can trigger ISR through its LPS, pseudobactin siderophore, or flagella (Meziane et al., 2005). In such situations, redundancy causes any of these determinants to be sufficient, yet not required, for induction. Hence, it can be assumed that many bacterial strains are potential inducers of systemic resistance in plants.
Table 1. Induction of systemic resistance by different ISR-eliciting bacteria on NahG plants (+: present; -: absent).

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Plant species/Pathogen</th>
<th>ISR</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus amyloliquefaciens</em> IN937a</td>
<td><em>Arabidopsis/Erwinia carotovora</em></td>
<td>+</td>
<td>Ryu et al., 2004b</td>
</tr>
<tr>
<td><em>Bacillus pumilus</em> SE34</td>
<td><em>Arabidopsis/P. syringae pv. maculicola</em></td>
<td>+</td>
<td>Ryu et al., 2003a</td>
</tr>
<tr>
<td></td>
<td><em>Arabidopsis/P. syringae pv. tomato</em></td>
<td>-</td>
<td>Ryu et al., 2003a</td>
</tr>
<tr>
<td></td>
<td><em>Arabidopsis/Cucumber mosaic virus</em></td>
<td>+</td>
<td>Ryu et al., 2004a</td>
</tr>
<tr>
<td></td>
<td><em>Tobacco/Peronospora tabacina</em></td>
<td>+</td>
<td>Zhang et al., 2002</td>
</tr>
<tr>
<td><em>Bacillus pumilus</em> T4</td>
<td><em>Arabidopsis/P. syringae pv. maculicola</em></td>
<td>+</td>
<td>Ryu et al., 2003a</td>
</tr>
<tr>
<td></td>
<td><em>Arabidopsis/P. syringae pv. tomato</em></td>
<td>+</td>
<td>Ryu et al., 2003a</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> GB03</td>
<td><em>Arabidopsis/Erwinia carotovora</em></td>
<td>+</td>
<td>Ryu et al., 2004b</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> 7NSK2</td>
<td><em>Tobacco/tobacco mosaic virus</em></td>
<td>-</td>
<td>De Meyer et al., 1999</td>
</tr>
<tr>
<td></td>
<td><em>Tomato/Botrytis cinerea</em></td>
<td></td>
<td>Audenaert et al., 2002</td>
</tr>
<tr>
<td></td>
<td><em>Tomato/ Meloidogyne javanica</em></td>
<td>+</td>
<td>Siddiqui and Shaukat, 2004</td>
</tr>
<tr>
<td><em>Pseudomonas chlororaphis</em> 06</td>
<td><em>Tobacco/P. syringae pv. tabaci</em></td>
<td>+</td>
<td>Spencer et al., 2003</td>
</tr>
<tr>
<td></td>
<td><em>Tobacco/Erwinia carotovora</em></td>
<td></td>
<td>Spencer et al., 2003</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> CHA0</td>
<td><em>Arabidopsis/Peronospora parasitica</em></td>
<td>+</td>
<td>Iavicoli et al., 2003</td>
</tr>
<tr>
<td></td>
<td><em>Tomato/Meloidogyne javanica</em></td>
<td>+</td>
<td>Siddiqui and Shaukat, 2004</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> WCS417</td>
<td><em>Arabidopsis/P. syringae pv. tomato</em></td>
<td>+</td>
<td>Pieterse et al., 1996</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> 89B61</td>
<td><em>Arabidopsis/P. syringae pv. maculicola</em></td>
<td>+</td>
<td>Ryu et al., 2003a</td>
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<tr>
<td></td>
<td><em>Arabidopsis/P. syringae pv. tomato</em></td>
<td>+</td>
<td>Ryu et al., 2003a</td>
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<tr>
<td></td>
<td><em>Arabidopsis/Cucumber mosaic virus</em></td>
<td>+</td>
<td>Ryu et al., 2004a</td>
</tr>
<tr>
<td></td>
<td><em>Tobacco/Phytophthora infestans</em></td>
<td>+</td>
<td>Yan et al., 2002</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> 90-166</td>
<td><em>Arabidopsis/P. syringae pv. maculicola</em></td>
<td>+</td>
<td>Ryu et al., 2003a</td>
</tr>
<tr>
<td></td>
<td><em>Arabidopsis/P. syringae pv. tomato</em></td>
<td>+</td>
<td>Ryu et al., 2003a</td>
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<td></td>
<td><em>Arabidopsis/Cucumber mosaic virus</em></td>
<td>+</td>
<td>Ryu et al., 2004a</td>
</tr>
<tr>
<td></td>
<td><em>Tobacco/P. syringae pv. tabaci</em></td>
<td>+</td>
<td>Press et al., 1997</td>
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</tbody>
</table>
Conclusions

Various rhizobacterial strains can promote plant growth by different mechanisms, as well as elicit induced systemic resistance (ISR) against several diseases in a strain/species-specific manner. ISR signaling in Arabidopsis, tobacco and tomato is almost always different from SAR signaling, as it does not require SA accumulation and is not associated with the induction of PRs. Both SAR and ISR are priming the plant for SA- and JA/ethylene-dependent defense-related gene expression, respectively, and are differentially active against biotrophic and necrotrophic pathogens; SAR and ISR can act additively against pathogens with intermediate lifestyles. Different bacterial determinants appear to be involved in the elicitation of ISR, making ISR a phenomenon of both scientific and practical interest.

References


Activity, chemistry and biosynthesis of cyclic lipopeptides produced by *Pseudomonas* species

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Abstract: Soil-borne diseases caused by oomycete and fungal pathogens are major yield-limiting factors in the production of food, fibers and ornamental crops. Biological control of plant diseases with antagonistic microorganisms offers an attractive alternative or supplement to currently used control measures. Studies in different laboratories have led to the identification of antagonistic strains of *Pseudomonas* species with biocontrol activity. The biocontrol activity of several of these *Pseudomonas* strains is determined, in part, by the production and secretion of metabolites with antibiotic activities. Among the antibiotic metabolites, cyclic lipopeptides (CLPs) have received considerable interest. CLPs are diverse molecules that contain a peptide moiety linked to a fatty acid tail. Specific biocontrol activity of CLPs against both oomycete and fungal pathogens has been reported and specificity is presumably related to the amino acid sequence and/or the length of the fatty acid tail. CLPs are produced by multi-enzyme complexes composed of several nonribosomal peptide synthetases with distinct modules. The current knowledge of the diversity and biosynthesis of multiple CLPs produced by antagonistic and plant pathogenic *Pseudomonas* species will be discussed.

Key words: *Pseudomonas*, cyclic lipopeptides, non-ribosomal peptide synthesis.

Introduction

Interactions between antagonistic microorganisms and plant pathogenic fungi have received considerable interest in the areas of microbial ecology, biological control, plant pathology and soil microbiology. When applied to seeds or planting material, antagonistic microorganisms offer an attractive alternative or supplement to currently used disease control strategies. Interest in biological control of plant pathogens by antagonistic microorganisms has been stimulated in recent years by trends in agriculture towards greater sustainability and public concern about the use of environmentally hazardous pesticides. There is now substantial evidence that metabolites including antibiotics, siderophores, and volatiles produced in situ by antagonistic bacteria play key roles in the control of various soil-borne plant pathogens (Raaijmakers *et al.* 2002).

Recent studies in our laboratory have led to the identification of several *Pseudomonas fluorescens* isolates with zoosporicidal activities (Souza *et al.* 2003). Zoospores of multiple oomycetes, including *Pythium* and *Phytophthora* species, were rendered immotile within 30 seconds of exposure to cell suspensions or cell-free culture supernatants of these *Pseudomonas* isolates, and subsequent lysis occurred within 60 seconds. One of these *Pseudomonas* isolates was identified as *P. fluorescens* strain R1SS101 (Souza *et al.* 2003). Application of cell suspensions of strain R1SS101 to soil or hyacinth bulbs provided significant protection against root rot caused by *Pythium intermedium* (Souza *et al.* 2003). Reverse-phase high-performance liquid chromatography of secreted compounds of R1SS101 yielded eight different fractions, five of which had surface activity and caused lysis of zoospores. Mass spectrometry and nuclear magnetic resonance analysis allowed the identification of the main constituent as a cyclic lipopeptide containing nine amino acids and a 10-carbon hydroxy fatty acid (Souza *et al.*, 2003). This compound was previously identified in a marine *Pseudomonas* sp. and was named Massetolide A (Gerard *et al.* 1997).
Cyclic lipopeptides

The production of cyclic lipopeptides (CLPs) has been reported for several *Pseudomonas* species, including plant pathogenic *P. syringae* (Bender et al. 1999). However, CLPs are also produced by a variety of other bacterial genera and fungi. CLPs constitute a large group of structurally related compounds. Examples of two structurally related groups of CLPs are listed in Figure 1. The common feature is a fatty acid coupled to the N-terminal part of a relatively short oligopeptide, which is cyclized to form a lactone ring between two amino acids. The compounds within these groups are very variable due to differences in fatty acid (length and modification), type, number and configuration of the amino acids, and organization of the lactone ring (Nybroe & Sørensen, 2004).

**Viscosin group**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
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</thead>
</table>

**Amphisin group**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
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</table>

Figure 1. Simplified primary structures of cyclic lipopeptides produced by *Pseudomonas* sp. (after Nybroe & Sørensen, 2004).

CLPs produced by *Pseudomonas* species constitute a complex group of compounds that have various biological functions. Some cyclic lipopeptides may function as chelating agent specific for cations like Ca$^{2+}$ and Mg$^{2+}$ (Grangemard et al. 2001). A physical property of CLPs is reducing the surface tension. As a biosurfactant, CLPs can be involved in wetting waxy surfaces, as was described for the virulence factor viscosin produced by plant pathogenic *P. fluorescens* (Laycock et al., 1991; Braun et al., 2001). In addition, biosurfactants may enable bacteria to get access to water-insoluble compounds. Finally, Nielsen et al. (2002) found a strong correlation between CLP production and surface motility of fluorescent pseudomonads. As mentioned before, CLPs also show antimicrobial activity. This interesting characteristic can be exploited in agriculture. Antimicrobial activity of CLPs has been reported in biocontrol against a range of gram-positive pathogenic bacteria, fungal and oomycete pathogens. For example, massetolide A and viscosin, two closely related cyclic lipopeptides, are both active against *Mycobacterium tuberculosis* (Gerard et al., 1997) and several oomycete pathogens like *Phytophthora* and *Pythium* (Souza et al., 2003). Viscosinamide, another cyclic lipopeptide belonging to the viscosin group (Figure 1), shows biocontrol activity against the fungal pathogen *Rhizoctonia solani* and the oomycete *Pythium ultimum* (Nielsen et al. 1999). One of the modes of action of CLPs reported for plant pathogenic *Pseudomonas* species is pore formation in cellular membranes followed by an efflux of cellular compounds and cell lysis (Agner et al., 2000; Dalla Serra et al., 1999).

In our research project, the focus is on two *Pseudomonas fluorescens* strains producing different cyclic lipopeptides. Strain R1SS101 produces massetolide A (Souza et al., 2003),
and the other strain produces viscosin. These two cyclic lipopeptides only differ in one amino acid in the peptide moiety (Figure 1). Tn-5 transposon mutagenesis libraries were constructed for both isolates to identify genes involved in the CLP biosynthesis. Subsequent characterization of CLP-deficient mutants yielded over 20 mutants (Souza et al., 2003, Raaijmakers et al. unpublished data). Sequence analysis of flanking regions of the transposon revealed that the transposons had integrated into several nonribosomal peptide synthetase genes and into genes related to regulation and secretion. Currently, the biosynthetic templates for massetolide A and viscosin are fully sequenced (De Kock et al. unpublished data). This knowledge can be used to further stimulate the production of these cyclic lipopeptides but can also be exploited to produce new cyclic lipopeptides with increased biocontrol activity.

Figure 2: Principle of cyclic lipopeptide biosynthesis by nonribosomal peptide synthetases. An operon with three genes (NRPS-1, -2, -3) coding for the synthetase subunits below the genes. Lines indicate the position of the modules, whereas the individual domains are shown as grey circles. (A, adenylation domain (activation of amino acid); T, peptidyl carrier protein domain (thiolation of amino acid); C, condensation domain (peptide bond formation between two amino acids); TE, thioesterase domain (cyclization)). The growing peptide chain is passed from left to right, until the linear product at the last T domain is cyclized by the TE domain. The β-hydroxy fatty acid is likely to be attached by the N-terminal C-domain (after Bruner et al., 2002)

**Nonribosomal peptide synthetases**

CLPs are produced on large, multi-functional nonribosomal peptide synthetases. The nonribosomal peptide synthetases are organized in domains that consist of modules (Figure 2). The modules can catalyse the reactions necessary for the formation of an elongating peptide product (Marahiel et al., 1997; Doekel and Marahiel, 2001). In brief, specific enzymatic domains recognize the amino acid substrates and activate them by adenylation. These unstable intermediates, which are associated with the enzyme, are subsequently stabilized by thioesterification carried out by a thiolation domain. Finally, peptide bonds are formed between activated precursors by a condensation domain. In addition to these domains,
specific parts of the enzyme may play a role in, for example, epimerization, cyclization reaction and the final release of the peptide product from the peptide synthetase. Several of the domains carry conserved amino acid motifs, which facilitate identification of these enzymes from sequence data.

Specific residues in the different domains of nonribosomal peptide synthetases determine the final structure and sequence of the amino acids in the peptide moiety of the CLP (Stachelhaus et al., 1999). These specific residues can be extracted from protein and domain alignments and can subsequently be used to predict the final structure and sequence of the peptide moiety of the CLP. Until now, little was known about the signature sequences of Pseudomonas nonribosomal peptide synthetases. Therefore, we analyzed annotated and unannotated Pseudomonas nonribosomal peptide synthetase sequences present in public databases supplemented with sequences we recently obtained from the synthetic templates for massetolide A and viscosin. Biochemical analysis of the CLPs produced by multiple, fully sequenced Pseudomonas strains, is currently ongoing to validate the chemical structures of the peptide moiety that are predicted on the basis of sequence data.

Conclusions

CLPs constitute a large group of structurally related compounds that differ in type, number and configuration of amino acids, organization of the lactone ring and length of the fatty acid tail. Besides diverse biological functions, CLPs can be exploited in the biocontrol of many pathogens. Biosynthesis and especially regulation of CLPs produced by antagonistic Pseudomonas species is not fully understood. Therefore, we investigate the biosynthetic pathways of two closely related cyclic lipopeptides Massetolide-A and viscosin. Bioinformatical analysis of protein sequences have already resulted in a detailed description of module and domain organization for each of the two synthetic templates. Additionally, this analysis allows the prediction of the sequence and structure of CLPs on the basis of genomic sequences. Major challenges for the future are to understand the biosynthesis of cyclic lipopeptides and its regulatory networks, and to apply this knowledge in durable biocontrol of plant pathogens.

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