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FOREWORD

Novel applications of molecular techniques and new insights into the molecular aspects of biocontrol of plant diseases are highlighted in this bulletin, which contains mini-reviews and short research papers presented at the workshop Molecular Approaches in Biological Control. This workshop was held at the Centre St. François in historic Delémont, Jura, Switzerland from 15-18 September 1997. This workshop was the fifth in a series on different topics in biocontrol and was cosponsored by the Working Group on Biological Control of Fungal and Bacterial Plant Pathogens of the International Organization for Biological Control - West Palaearctic Regional Section (IOBC/wprs), the Swiss Society for Phytiatry, and the European Foundation of Plant Pathology (EFPP).

This was a truly international meeting with 38 lectures and 28 posters presented by 73 scientists from Belgium, Canada, China, Denmark, France, Germany, Ireland, Israel, Italy, the Netherlands, New Zealand, Poland, Rumania, Spain, Sweden, Switzerland, Turkey, the United Kingdom, the United States, and Yugoslavia. Special sessions and discussions focused on:

- Mechanisms of action with bacterial agents in the rhizosphere, the phyllosphere, and postharvest - lead by invited speakers Mark Wilson, Auburn University, AL, USA and Steve Hill, Novartis, NC, USA
- Mechanisms of action with fungal agents - lead by invited speaker Matteo Lorito, University of Naples, Italy
- Induced resistance - lead by invited speaker L.C. van Loon, Utrecht University, The Netherlands
- Strategies to improve biocontrol - lead by invited speaker Ilan Chet, the Hebrew University of Jerusalem, Israel
- Microbial ecology and monitoring - lead by invited speakers Jos Raaijmakers, Washington State University, WA, USA and Tim Denny, University of Georgia, GA, USA
- Risk assessment - lead by invited speaker John Whipps, Horticultural Research Institute, United Kingdom

Lively discussions between representatives of academia, extension, and industry covered the range of fundamental research to practical applications, addressing research goals and ethical and political issues facing biocontrol and biotechnology in the near future. The zest of this meeting is captured in the papers compiled in this bulletin, which represents the cutting edge in international biocontrol research.

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We thank the ETH Phytomedizin group (http://www.pa.ipw.agrl.ethz.ch/pathology), the Sisters of the Centre François, and all the participants whose help made this an exceptionally enjoyable and memorable experience for everyone. A balance of scientific and social programmes (walks around the old town, relaxing in the meditation garden, wine and chocolate aperós, a canoe trip down the Doubs river that left most of us looking as though we'd been swimming in the river!) created the atmosphere for founding friendships and collaborations which we hope will continue.

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Molecular investigations into mechanisms in the biological control of postharvest diseases of citrus

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Abstract

Pseudomonas syringae strains ESC-10 and ESC-11 produce syringomycin and control green and blue molds of citrus caused by *Penicillium digitatum* and *P. italicum*, respectively. Although syringomycin inhibits the fungi in vitro, it is not known what role syringomycin production plays in disease control. A *lacZ* reporter gene system was used to determine the influence of orange and lemon rinds on production of syringomycin E in vitro. The *syrB* biosynthesis gene disrupted by *lacZ* was used to replace the wild-type allele in strains ESC-10 and ESC-11, generating syringomycin E mutants. In cultures inoculated with the *syrB* mutant of strain ESC-11, β-galactosidase activity in a minimal medium (SRM) did not increase when lemon or orange flavedo tissue were added, indicating that these tissues do not induce production of syringomycin in this strain. However, in cultures inoculated with the *syrB* mutant of strain ESC-10, β-galactosidase activity was higher in media containing fruit albedo tissue after 4 days incubation. We are currently using the reporter-gene system to determine if syringomycin E is produced by these strains in wounds on lemons or oranges.

Introduction

Postharvest diseases impose a significant economic burden on citrus growers. In California, losses due to postharvest decay are estimated to be worth millions of dollars annually (Eckert and Eaks 1989). Postharvest diseases have the opportunity to destroy the harveted fruit during the time between its harvest and consumption. In addition to the cost of growing lemons and oranges, the cost the of harvest, transport, and postharvest treatment are also lost when the fruit is destroyed prior to consumption. Additional costs arise when entire cartons of fruit need to be repackaged due to unsightly spread of spores from one or a few diseased fruit. In California, *Penicillium digitatum* (Pers.:Fr.) Sacc., and *P. italicum* Wehmer causing green mold and blue mold, respectively, and *Geotrichum citri-aurantii* (Ferraris) R. Cif. and F. Cif. (syn. *G. candidum*) causing sour rot, are responsible for a large portion of the loss due to postharvest decay in the citrus industry (Eckert and Ogawa 1985). Under conventional agricultural practices, a number of synthetic chemicals, including imazalil, thiabendazole, and sodium O-phenylphenol, have been used to combat postharvest losses in citrus (Eckert and Ogawa 1985). Alternatives to these chemicals are currently being sought because of the high level of resistant isolates present in fungal populations, the very high cost of registering new chemicals, stricter regulations on use of synthetic chemicals, and increasing public's awareness and perception of dietary risk. Biological control of postharvest diseases is one

alternative that is being evaluated by researchers worldwide (Janisiewicz 1988, Wilson et al. 1991). In 1995, two biological control products were accepted for postharvest use on citrus by the Environmental Protection Agency of the United States. BioSave 1000™ (EPA reg. no. 64296-7) and Aspire™, and (EPA reg. no. 5538-29) contain the bacterium *Pseudomonas syringae* strain ESC-10 and the yeast *Candida oleophila* strain I-82, respectively, as the active ingredients in these products. These treatments have also been approved for use on organically grown fruits in California. In addition to the treatment of citrus, strain ESC-10 is also used to control postharvest diseases on pomes. A related product, BioSave 110 contains *P. syringae* strain ESC-11 and is registered for use on apple and pear. *Pseudomonas syringae* strains ESC-10 and ESC-11 were originally isolated from pear and apple, respectively. These bacteria and *C. oleophila* strain I-82 are the only agents which have been commercialized for control of postharvest diseases in the U.S.

Since 1995, we have worked with the bacterial biological control products in an attempt to understand how they control plant diseases. The green and blue mold pathogens exploit wounds to infect fruit. The bacterial agents survive in wounds where they are needed to protect the fruit, but populations quickly decline on fruit surfaces. Strain ESC-10 consistently controls disease better than strain ESC-11 and differences in survival of the bacteria in wounds on lemon do not account for these differences (Bull et al. 1997). Strain ESC-10 consistently controls green and blue molds on lemons better than on oranges but again differences were not due to differences in survival of the bacterium in wounds on the different fruit (Bull et al. 1997). BioSave 1000 prevents infection but is a poor eradicant (Smilanick et al. 1995).

In addition to describing the efficacy of using these control products, we are studying the mechanism(s) by which these organisms operate to control disease, in order to more effectively integrate their use in biorational control measures. *Pseudomonas syringae* strains ESC-10 and ESC-11 are related to the pathogen *P. syringae* pv. *syringae* which causes disease on a variety of hosts including citrus. However, strains ESC-10 and ESC-11 did not cause disease in host range studies conducted in support of the EPA registration (Smilanick et al. 1996). In addition, strain ESC-11 does not illicit the hypersensitive reaction on tobacco indicating that it may not be a pathogen on any plant (Smilanick et al. 1996). Pathogenic *P. syringae* pv. *syringae* strains produce a class of small cyclic lipoproteins called syringomycins (Quigley and Gross 1994). These compounds are involved in virulence and are general biocides inhibiting a wide range of fungal pathogens, including the fungi important to postharvest diseases of citrus (Sinden et al. 1971). Quigley and Gross (1994) demonstrated that many *P. syringae* strains for which no host is known have genes for syringomycin production and export and produce forms of syringomycin. Antifungal compound(s) are produced by strains ESC-10 and ESC-11 in vitro (Bull et al. 1998, Wadsworth et al. 1996). Syringomycin E is the major component of the antifungal activity of these strains (Bull et al. 1998, Wadsworth et al. 1996). Syringomycin E, purified from cultures of either ESC-10 or ESC-11, controlled green mold on lemons (Bull et al. 1998). Although this study indicated that syringomycin might be involved in biological control of green mold on lemons, there is no evidence that syringomycin is produced in wounds on citrus where it would be needed to control the disease. Syringomycin and similar compounds have not been isolated or detected from plant surfaces. We therefore initiated these experiments to determine if genes involved in the biosynthesis of syringomycin are expressed in the presence of albedo tissue from lemons and oranges.

Materials and Methods

Syringomycin E mutants of *P. syringae* strains ESC-10 and ESC-11 have a promoterless *lacZ* gene inserted behind the promoter of the *syrB* gene (syringomycin synthetase gene) and no longer produce syringomycin. Bacterial strains were grown overnight in nutrient broth (NB). A separate culture initiated from a single colony was grown for each replication. NB cultures (2 ml) were centrifuged, resuspended in water, and recentrifuged. Cells were resuspended in 100 µl of sterile water. The cells were used to inoculate 20 ml of syringomycin minimal medium (SRM-af; Mo and Gross 1991b), SRM amended with 100 µM arbutin and 0.1% fructose, or SRM made from albedo juice of either orange or green and yellow lemons. Albedo juice was prepared by removing the flavedo of the fruit with a knife and collecting 28 g of the white albedo tissue. The albedo tissue was macerated in a Waring Blendor with 400 ml of distilled water. The juice was then centrifuged to remove cellular debris and was filtered to clarify the solution.

Activity of *syrB* was measured by assaying and calculating β-galactosidase activities by standard methods (Stachtel et al. 1985). Gene activity was measured from cultures after four days incubation at 27 °C without agitation. There were five replications of each medium, the experiments were conducted twice, and *syrB* expression for each strain was monitored in separate experiments.

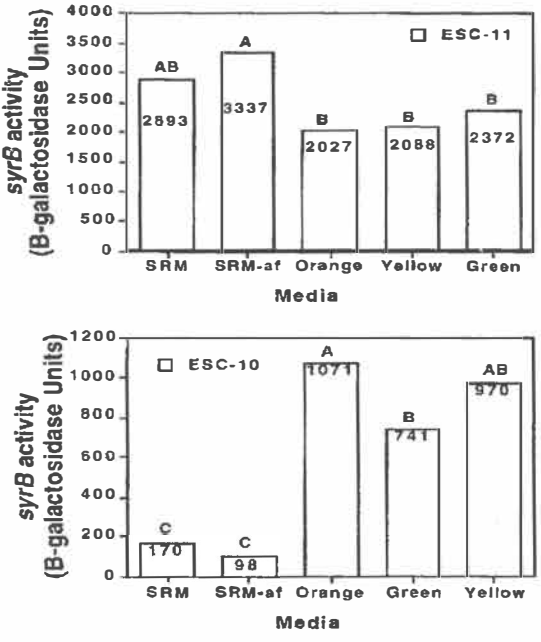

Fig. 1: Expression of syrB gene from *P. syringae* strains ESC-11 and ESC-10. The strains used contained a promotorless lacZ in the syrB gene. After 4 days incubation at 27 °C without agitation, syrB expression was estimated by measuring β-galactosidase activities by standard methods (Stachel et al. 1985). SRM is a minimal medium used for syringomycin production which was ammended with arbutin and fructose (SRM-af; Mo and Gross 1991b) or was made from macerated albedo tissue from oranges (Orange), and yellow (Yellow) or green (Green) lemons. Values represent the means of five replications. Treatments with similar letters were not significantly different at ($P = 0.05$) according to Student-Newman-Keuls test.

Results

After 4 days incubation, inoculation with the *syrB* mutant of strain ESC-11 resulted in β-galactosidase activities which were over 2000 Units in all the media tested (Fig. 1). β-galactosidase activity in a minimal medium (SRM) did not increase when lemon or orange flavedo tissue were added. There was no influence of fruit on the level of activity determined. Media amended with orange, or yellow or green lemons had similar levels of activity. Additionally, the number of units of activity produced by this strain in medium amended with arbutin and fructose was greater than the level of production in mediumamended with fruit albedo.

In cultures inoculated with the *syrB* mutant of strain ESC-10, β-galactosidase activity was higher in media containing fruit albedo tissue than in the media not amended or amended with arbutin and fructose (Fig. 1). Amendments with fruit albedo tissue resulted in gene expression that was 4 to 6 times higher than the expression observed in unamended SRM. Gene expression was significantly higher in SRM medium amended with orange albedo tissue than in SRM amended with green lemon albedo tissue. No difference was detected between expression detected in media amended with green verses yellow lemon albedo tissue. No differences were detected in gene expression in cultures grown in SRM or in SRM amended with arbutin and fructose.

Discussion

The role of syringomycin E in biological control of postharvest diseases on citrus by *P. syringae* strains ESC-10 and ESC-11 is unclear. Several lines of evidence indicate that syringomycin E may be involved, however, some data suggest that production of syringomycin E is not needed for control. Production of syringomycin E in wounds on citrus is a prerequisite to its being involved in disease control of wound pathogens. Because syringomycin E integrates into cell membranes and has never been detected from plant tissue, alternative methods for detecting production of syringomycin E in situ have been developed (Mo et al. 1991a). Reporter genes have been used effectively to monitor production of compounds which are not easily detected from environmental samples. We report here initial experiments determining the effect of citrus tissue on production of syringomycin. These are the first experiments in which citrus tissue is examined for its ability to induce production of syringomycin E by strains ESC-10 and ESC-11.

In culture, ESC-11 consistently produces higher concentrations of syringomycin E than does strain ESC-10 (Bull et al. *unpublished data*). Levels of expression of the *syrB* gene were consistent with this finding. Although gene expression by strains ESC-10 and ESC-11 were not compared in the same experiment, the expression of the *syrB* gene was 1.9 to 34 times higher in strain ESC-11 than in ESC-10, depending on the medium used. If syringomycin E is involved in biological control by these strains, one might hypothesize that strain ESC-11 would provided better control than strain ESC-10. However, in numerous experiments, strain ESC-10 consistently reduces the incidence of green mold on lemons to a greater extent than does strain ESC-11 (Bull et al. 1997). Therefore, if syringomycin E is involved in biological control by these strains, then syringomycin E production in vitro may be different than production in the infection court. The result with media amended with citrus albedo tissue may be an indication of the effect of citrus on gene expression. In addition, these experiments will be useful in comparison with syringomycin production in the albedo amended media for determining the significance of β-galactosidase activities expressed in wounds on fruit.

Expression of *syrB* in strain ESC-11 was less in media amended with citrus albedo tissue when compared to media amended with arbutin and fructose. Additions of the plant signal molecules arbutin and fructose stimulate expression of syringomycin in many *P. syringae* pv. *syringae* strains including ESC-11 (Bull et al. *unpublished data*, Mo et. al. 1997a). These studies indicate that for strain ESC-11 sugars and potential signal molecules from albedo tissue do not result in the same level of induction as the combination of arbutin and fructose. Although these results do not demonstrate gene activities in the infection court, they do indicate that the presence of citrus albedo tissue may reduce the expression of *syrB* by strain ESC-11.

In contrast, the addition of citrus albedo tissue to the minimal medium increased the expression of *syrB* by strain ESC-10. Expression of *syrB* was 2.4 to 6.3 times greater in media amended with citrus albedo than in unamended media. Differential induction of syringomycin production by strains ESC-10 and ESC-11 in response to citrus albedo tissue may partially explain why ESC-10 is able to control postharvest pathogens to a greater extent than strain ESC-11 even though ESC-11 produces higher concentrations of syringomycin E in vitro than does strain ESC-10.

In strain ESC-10, expression of *syrB* was higher in the medium amended with orange albedo than the medium amended with green lemon albedo. This result is inconsistent with the finding that strain ESC-10 controls green mold to a greater extent on lemons (especially green lemons) then on oranges (Bull et al. 1998). At this time we have no explanation for this result.

In these experiments we have demonstrated expression of *syrB* in the presence of citrus albedo tissue. It is essential to determine if syringomycin E is produced in the infection court on citrus as part of the evidence for its involvement in biological control of green mold. In addition, production of syringomycin E by microorganisms applied as biological control agents on consumable products may be of concern to consumers. Because expression of *syrB* in the presence of albedo suggests, but does not prove, that the product will be present in the infection court, we are currently evaluating expression of *syrB* in wounds on citrus.

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Mechanisms in the biological control of *Pseudomonas syringae* pv. *syringae* by *Pantoea agglomerans*

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Abstract

Pseudomonas syringae pv. *syringae* is the primary causal agent of the basal kernel blight disease of barley in Montana. Pulsed-field gel electrophoresis was used to obtain macrorestriction fingerprints of restriction enzyme-cut DNA of field isolates of *P. syringae* pv. *syringae*. Strains within the *syringae* pathovar were diverse within a field population, but showed similar banding patterns when isolated from the same barley cultivar. Despite the fact that genomic fingerprints demonstrated heterogenous *P. syringae* pv. *syringae* populations, biocontrol treatment using *Pantoea agglomerans* applied prior to the infection window at early milk stage in the field or prior to pathogen attack in greenhouse tests provided 60 to 100% reductions in basal blight infected seeds. Multiple mechanisms seem to be involved in the biological control of *P. syringae* pv. *syringae* pathogens by *P. agglomerans*. We suggest that the primary mechanisms include (i) competition for iron and other nutrients, (ii) competition for space as demonstrated by scanning electron micrographs of the kernel surface and population dynamics studies with antibiotic resistant mutants, and (iii) induced systemic resistance by the host plant barley.

Introduction

We previously reported about the etiology and significance of basal kernel blight of barley caused by *Pseudomonas syringae* pv. *syringae* (Martinez-Miller et al. 1998). Since the use of bactericides on cereal grains is not considered practical on a commercial scale, the only control measure to date has been the avoidance of irrigation during the most susceptible kernel developmental stage at soft dough (EC 85) and the use of less susceptible cultivars. Over the last 3 years we have developed an effective biological control system based on the application of *Pantoea agglomerans* prior to the *P. syringae* pv. *syringae* infection window. Field experiments in 1994 and 1995 revealed a suppression of 60 to 80% kernel blight disease. In greenhouse studies, diesease control was as high as 80 to 100% (Braun et al. 1997). Application timing is critical. However, a single *P. agglomerans* application prior to the expected infection window is sufficient since log 7 CFU/kernel populations are established while pathogen populations are kept reduced by approximately log 2 CFU/ kernel. Here we report on the diversity of *P. syringae* pv. *syringae* field populations. We further investigated competition for iron and space, and induced resistance as possible mechanisms in the complex interaction between biocontrol agents, pathogens, and the host plant.

Materials and Methods

Genetic diversity of *P. syringae* pv. *syringae* field isolates was evaluated by comparing genomic DNA-profiles of isolates from different cultivars showing symptoms of basal blight in

1994 and 1995. Pulsed-field gel electrophoresis (PFGE) provides information on population structure of a particular pathogen in addition to phylogenetic relationships between strains from the same or different cultivars. This information is essential for the use of potential biocontrol agents since efficacy has to be determined for the pathogen in question and its derivatives. Culture preparation for PFGE was done according to Megeed and Sherwood (1995). Agar plugs containing lysed bacterial cells were digested with 40 U of XbaI [TCTAGA] or 10 U of SpeI [ACTAGT], respectively, stored in 0.5 M Na-EDTA (pH 8.0) at 4 C. PFGE was carried out using the BIO-RAD CHEF DR-II system in a 1% agarose gel. Lambda DNA concatomers (BIO-RAD Laboratories) were used as molecular size markers. A voltage of 200 V was applied for a running time of 22 h for XbaI and 23 h for SpeI in 0.5x TBE buffer at 18 C. Optimal separation of DNA fragments in the regions between 100 to 300 kb was achieved by ramping the switch time from 8 to 20 s during the running time. After the run, DNA was visualized by staining with ethidium bromide and UV trans-illumination. Dendrograms were obtained after cluster analysis of XbaI and SpeI DNA fragment patterns using percent dissimilarity values (PD-Index) and the unweighted pairgroup method (Ludwig and Reynolds 1988).

Competition for iron in the barley phyllosphere was evaluated in a greenhouse experiment. Plants (cv. B 2061) were treated with: (i) 100 µM $FeCl_3$ alone, (ii) the biocontrol strain Eh 239 + 100 µM $FeCl_3$ 3 days prior to inoculation with *P. syringae* pv. *syringae* 552 at soft dough stage, (iii) *P. syringae* pv. *syringae* 552 alone (positive check), (iv) H_2O (negative check), (v) *P. syringae* pv. *syringae* 552 D as heat killed pathogens, (vi) the systemic resistance inducer Actigard® (CGA-245704), (vii) the biocontrol strain Eh 239 alone, and (viii) the strain Eh 239 at EC 49 (last boot stage before heading to explore the possibility of earlier protection by the biocontrol strain). All bacteria were sprayed to runoff on barley spikes in a water solution containing a concentration of log 7 CFU/ml and 0.025% Tween 20. Actigard® had a concentration of 100 ppm active ingredient. Plants were incubated in a mist chamber for 72 h providing about 95% rh after biocontrol strain and chemical application and again after inoculation with *P. syringae* pv. *syringae* 552. Basal kernel blight percentage was evaluated after harvest by dividing the amount of blighted kernels by the amount of total kernels obtained from three barley heads per replication. The experiment was conducted as a completely randomized design with four replications per treatment, and multiple comparisons between treatment means are based on the LSD test at $P < 0.05$.

Competition for space was investigated. Scanning electron micrographs of the barley kernel surface were analyzed 7 days following inoculation with the pathogen, *P. syringae* pv. *syringae* 522, and biocontrol strain Eh 460 at soft dough stage and incubation in a mist chamber at 18 to 20 °C for 72 h. Kernels were cut longitudinally mounted on specimen holders, sputter coated with 25 nm Au/Pd and examined with a JEOL 6100 scanning electron microscope at 8 to 10 KV.

Induced resistance was evaluated by spraying 100 ppm Actigard® onto barley heads at soft dough stage 3 days prior to inoculation with *P. syringae* pv. *syringae* 552 or *P. syringae* pv. *syringae* 793. Additional treatments included biocontrol strain Eh 460, Eh 237, and Eh 234. Barley inoculation, incubation, and percent kernel infection were evaluated as described above. A second more classical approach was conducted to compare induced systemic resistance in cv. B 2601 after treatment with Actigard® and biocontrol agents. Treatments consisted of Eh 239, 100 ppm Actigard® and H_2O (= non-induced control), and were applied on the entire upper leaf surface of the first true-leaf of 6 week old barley plants using a sterile cotton swab. Leaves were covered after application with pollination bags to avoid spread of

biocontrol agents. The second, third, and fourth leaves were challenge-inoculated three days later with a log 7 CFU/ml suspension of pathogenic *P. syringae* pv. *syringae* 552. *Pseudomonas syringae* pv. *syringae* 552 was syringe-injected into a marked 2-cm area on the lower leaf surface of three leaves per plant until water-soaking became visible. Plants were incubated in a mist chamber for 36 h. Evaluation of necrotic lesions expanding beyond the marked area were rated as positive infection 6 days after challenge-inoculation. Percent infection was calculated by counting infected versus non-infected leaves per plant. The experiment consisted of three plants per pot with three pots per treatment (n = 9).

Results

Genomic fingerprinting resulted in 5 to 10 DNA fragments between 100 to 300 kb for SpeI and 4 to 14 bands for XbaI, depending on the strain. Different pathovars revealed differences in their banding pattern. Strains within the syringae pathovar were still diverse (50 to 80% similarity), but showed higher similarity (100%) in 71% of the strains isolated from the same cultivar. The dendrogram (Fig. 1) shows the phylogenetic relationship between 14 *P. syringae* pv. *syringae* strains isolated from five barley cultivars in 1995. The cluster level is based on the PD-Index (percent dissimilarity), in which a value of 0 indicates 100% similarity between strains and a value of 1 indicates 0% similarity.

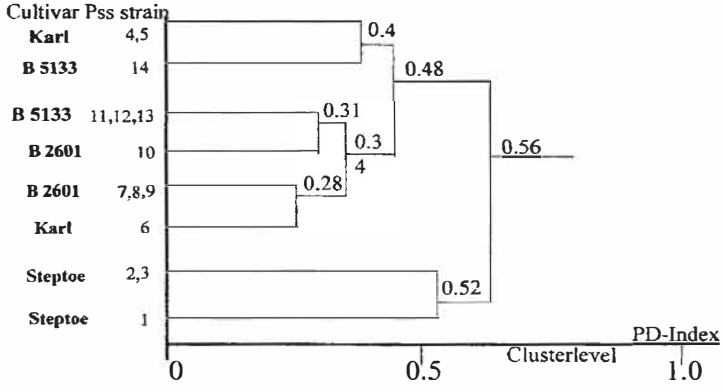

Fig. 1. Dendrogramm of dissimilarity values of macrorestriction DNA-fragment patterns for *Pseudomonas syringae* pv. *syringae* (PSS) strains from basal blight kernels of different barley cultivars. The cluster analysis was calculated from the percent dissimilarity (PD-value) of the cumulative SpeI and XbaI fingerprints by using the unweighted pair group method.

Influence of different barley head treatments on the percent of basal kernel blight infection, including competition for iron is presented in Fig. 2. The most effective treatment in this experiment was the application of the biocontrol strain Eh 239 three days prior to pathogen inoculation (87% disease reduction). The use of Eh 239 at EC 49 (82%) was not statistically different from the previous treatment, suggesting that biocontrol application can be done at earlier plant stages such as before heading. This result is also in accord with a possible induction of host plant resistance. The chemical inducer Actigard® provided control

levels of 73% without having a direct affect on the pathogen population (0 to 100 ppm active ingredient tested). The application of iron as $FeCl_3$ in combination with the biocontrol strain Eh 239 resulted in only 57% disease control, suggesting that competition for iron may be involved in the biocontrol-pathogen interaction, and confirming plate bioassays, in which zones of pathogen inhibition were smaller when 10 µM $FeCl_3$ was included in the minimal medium. However, even the $FeCl_3$ treatment alone resulted in 54% disease reduction similar to heat-killed pathogens, indicating again the possibility of induced resistance. 10 µM $FeCl_3$ amendment to minimal media did not show any negative effect on bacterial growth, while our results with 100 µM $FeCl_3$ were not conclusive.

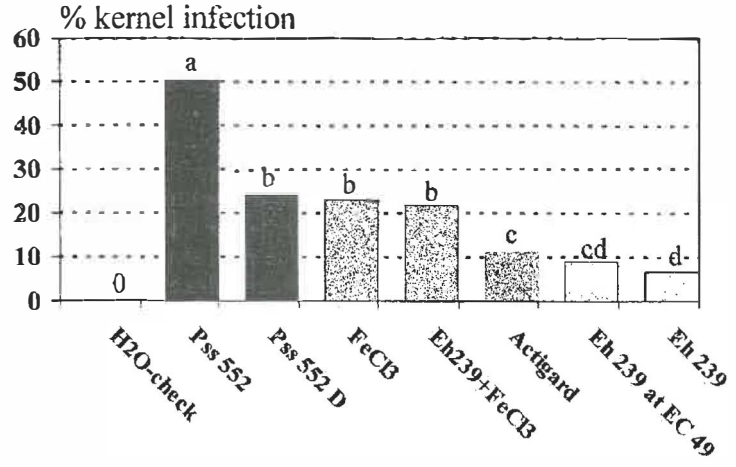

Fig. 2. Influence of *Pantoea agglomerans* biocontrol strain Eh 239 applied 3 days prior to *Pseudomonas syringae* pv. *syringae* (PSS) 552 at soft dough stage and before heading (EC 49), the systemic resistance activator Actigard® applied at soft dough, iron chloride alone and in combination with the biocontrol strain Eh 239, and heat-killed *P. syringae* pv. *syringae* 552 D on the basal kernel blight disease of barley caused by *P. syringae* pv. *syringae* 552. Means followed by the same letter are not significantly different at $P < 0.05$ according to Fisher's LSD test.

Competition for space was investigated. The scanning electron micrographs demonstrated that both pathogen and biocontrol agents occupied the same niches on the kernel surface 7 days after inoculation. Prefered sites appeared to be the base of trichomes and areas close to stomata.

Induced systemic resistance was investigated. Actigard® provided similar levels of basal kernel blight disease reduction (95 to 97%) as all biocontrol strains, which reduced infection levels by 72 to 100%, depending on which strain was tested and which pathogen strain was used for inoculation (*P. syringae* pv. *syringae* 552 or *P. syringae* pv. *syringae* 793, Fig. 3). The results of the classical induced resistance experiment are summarized in Table 1.

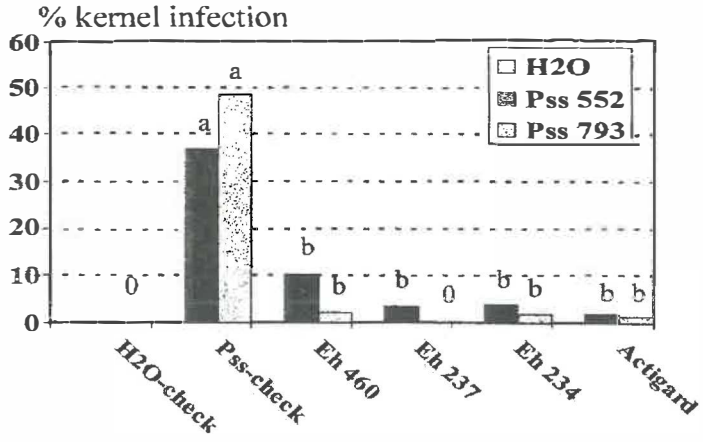

Fig. 3. Influence of *Pantoea agglomerans* biological control strains Eh 460, Eh 237, Eh 234, and Actigard®, a systemic induced resistance activator applied at soft dough stage prior to pathogen inoculation on the basal Kernel blight disease caused by *Pseudomonas syringae* pv. *syringae* 552 or *Pseudomonas syringae* pv. *syringae* 793, respectively. Means followed by the same letter are not significantly different at $P < 0.05$ according to Fisher's LSD test.

Table 1. Effect of *Pseudomonas syringae* pv. *syringae* 552 challenge-inoculation on the development of leaf necrosis (% infection) after application of the systemic resistance inducer Actigard® and the biocontrol bacterium *Pantoea agglomerans* Eh 239, respectively, on the first leaf of 6 week old barley plants.

Treatment	% infection with pathogen based on leaf necrosis	% disease reduction
Non-induced control	79.8[z]	-
Actigard®	39.5	50.5
Eh 239	43.4	45.6

[z] Means in a column represent values of three pots with three plants. For each plant, three leaves were evaluated per treatment.

Discussion

Results of these and previously reported experiments suggest that multiple mechanisms are involved in the biological control of *P. syringae* pv. *syringae* by *P. agglomerans*. The addiditon of 100 µM $FeCl_3$ in the barley phyllosphere limited the effectiveness of Eh 239. Since both *P. syringae* pv. *syringae* and *P. agglomerans* produce siderophores perhaps the one with the stronger chelators wins the battle for iron in an iron limited environment (Bull et al. 1994). The effectiveness and fitness of siderophore-minus mutants of Eh 239 after ethylmethylsulfonate treatment is currently under investigation. Competition for space appears to be involved in the interaction since scanning electron micrographs indicate that

P. syringae pv. *syringae* and *P. agglomerans* prefer the same microhabitats, mainly the sites around stomata and trichome bases. Actigard® and *P. agglomerans* in classical induced resistance studies revealed control of diseases caused by *P. syringae* pv. *syringae*. While this was done on leaves, studies on the reduction of basal kernel blight on barley heads are currently ongoing. The possibility that cell density-dependent virulence genes in *P. syringae* pv. *syringae* might not be expressed if the population is kept below a critical threshold (for autoinducer production) in the presence of *P. agglomerans* should be considered. *Pseudomonas syringae* pv. *syringae* is known to live in the phyllosphere without causing symptoms until epiphytic populations increase dramatically in association with disease development or until plant signals are released. This hypothesis is supported by the fact that *P. agglomerans* reduces *P. syringae* pv. *syringae* population densities in vitro and in vivo.

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Genetics of pantocin A and pantocin B production and their role in biocontrol of the fireblight pathogen, *Erwinia amylovora*

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Abstract

Two cosmid clones, pCPP702 and pCPP704, from a genomic library of *Pantoea dispersa* Eh318 carry the DNA responsible for the synthesis of pantocin A and pantocin B, respectively. These two antibiotics are active against *Erwinia amylovora* in vitro. The biosynthetic genes for pantocin A, designated *paaA*, *paaB* and *paaC*, are clustered and span 2.7 kb of DNA. The biosynthetic region for pantocin B is 19 to 20 kb in size, based on the analysis of transposon insertion data in a subclone of pCPP704, called pCPP719.

Eh318 has been rated as an excellent biocontrol agent of the fireblight pathogen, *E. amylovora*, in research orchard trials. The role of the two antibiotics in biocontrol was assessed by determining the relative disease reducing ability of mutants of Eh318 with transposon insertions in specific biosynthetic genes for pantocin A and pantocin B. Biocontrol ability of the strains was assessed in immature pear fruit in the laboratory and in apple blossoms on container-grown trees in a controlled environment chamber. Overall, the wild-type strain Eh318 was more effective than mutants affected in antibiotic production, thus indicating roles for pantocin A and B in biocontrol.

Introduction

Pantoea dispersa and *P. agglomerans* (syn. *Erwinia herbicola*, Holt et al. 1994) are common inhabitants of the phyllosphere. *Pantoea* spp. have been proposed as biocontrol agents for fire blight, a disease of rosaceous plants, caused by *E. amylovora*. *Pantoea* sp. is considered to act through the production of antimicrobial compounds (Vanneste et al. 1992), or through competition for nutrients or sites on the stigmas of blossoms (Hattingh et al. 1986, Wilson et al. 1992), where the bacteria multiply (Thomson 1986). *Pantoea dispersa* strain Eh318 has been effective against *Erwinia amylovora* in research orchard trials. It produces two distinct antibiotics, named pantocin A and pantocin B, that inhibit the growth of *E. amylovora* on plates (Wright 1997); formerly they were called antibiotic 717 and 719, respectively (Wright and Beer 1996). A genomic cosmid library of Eh318 DNA, constructed in *Escherichia coli* DH5α had been screened for antibacterial activity to *E. amylovora*, strain Ea273 (Wright 1997). Two cosmids, pCPP702 and pCPP704, had distinct restriction enzyme and DNA of pCPP702 did not hybridize with DNA of pCPP704 (Wright 1997). The two clones include the biosynthetic machinery for pantocin A and pantocin B, respectively (Wright 1997).

The present work was aimed at delineating the biosynthetic genes for the two antibiotics and exploring the role of antibiotics in biological control of fire blight in two in planta niches, immature pear fruit and in apple blossoms.

Materials and Methods

Bacterial strains were routinely cultured in Luria-Bertani (LB) medium (Sambrook et al. 1989) at 28 °C (*E. amylovora* and *P. dispersa*) or at 37 °C (*E. coli*). Antibiotic production was assayed either on glucose-asparagine medium (GA) (Wodzinski et al. 1987) or on *E. coli* minimal medium (EcMM) (Wright 1997), by either the chloroform assay or the live assay (Wright 1997). Plasmid and cosmid DNA was isolated and manipulated following standard procedures (Sambrook et al. 1989). Smaller clones were created from pCPP702 and pCPP704 that conferred on *E. coli* the ability to produce pantocin A and pantocin B, respectively. The smallest clone conferring pantocin A production to DH5α (called pCPP717) was analyzed further by nested deletion analysis. The insert DNA of pCPP717 was sequenced by the Cornell University Biotechnology Service Facility using the Taq dye terminator cycle sequencing method on a model 373 Strech Sequenator (Applied Biosystems, Perkin-Elmer, Inc., Norwalk, CN, USA). The deduced amino acid sequences were compared to known proteins in the database. Furthermore, a hydrophilicity profile was generated for the predicted product of one gene. DNA sequence data was assembled and analyzed by using the programs of the DNAStar Lasergene Software Package (Madison, WI, USA) and GCG, Inc. (Madison, WI, USA).

Mutagenesis of plasmids carrying portions of the insert DNA of pCPP702 or pCPP704 was done with transposons Tn*5*, MudI1734, mini-Tn*5Cm* and Tn*5gusA1*. Two of the transposon insertions were marker-exchanged into Eh318 to create pantocin-deficient mutants. Eh318 also was subjected to direct mutagenesis with Tn*5* by conjugating the transposon-containing plasmid pSUP2021 into Eh318, and subsequently "curing" it while selecting for kanamycin resistance in low-phosphate medium (Roeder and Collmer 1985). Insertions that produced an antibiotic-defective phenotype were mapped. Three of these mutants and marker-exchange mutants of Eh318 later were employed in bioassays to determine the relative contribution of antibiotics to biological control activity. To determine the direction of transcription of genes involved in pantocin B synthesis, GUS activity assays were performed on *E. coli* DH5α(pCPP719::Tn*5gusA1*) carrying insertions in different positions, according to the procedure described by Wei et al. (1992).

Two bioassays were employed to assess the role of pantocin A and B in biological control of fire blight, the immature pear fruit assay (Beer et al. 1984) and an apple blossom test in a controlled environment chamber (Beer 1990, Wright and Beer 1996). The conditions, general procedures for the assays, and their statistical treatment have been described previously (Wright and Beer 1996). The protective ability of the marker-exchange mutants of Eh318 was tested in both pear fruit and apple blossoms. The protection conferred by three Tn*5* mutants, which had been developed by direct mutagenesis of Eh318, was assessed only on immature pear fruit.

Results

The smallest clone of pCPP702 that conferred on *E. coli* the ability to produce pantocin A, pCPP717, consists of 3.9 kb of Eh318 DNA cloned into pBluescript. Data from deletion analysis of pCPP717 and mapping of transposon insertions indicated that only 2.7 kb of the insert DNA is required for pantocin A synthesis. Within this region lie three predicted open reading frames, designated *paaA*, *paaB* and *paaC*. The predicted *paaA* product has 50-60% end-to-end amino acid sequence similarity (Needleman-Wünsch algorithm) to several biosynthetic enzymes. Two are active in the synthesis of the cofactors thiamin and

molybdopterin of *E. coli,* while another is involved in the synthesis of microcin C7, a heptapetide antibiotic produced by *E. coli* (González-Pastor et al. 1995). All these enzymes share a dinucleotide binding fold motif in the central part of the protein (Wierenga et al. 1986). The hydrophilicity profile of the predicted *paaC* gene product revealed 10 strongly hydrophobic regions, predicted to be membrane-spanning α-helices.

The smallest subclone obtained from pCPP704 that conferred on *E. coli* the ability to produce pantocin B, pCPP719, carries 27.4 kb of Eh318 DNA. Mapping of mini-Tn*5Cm* and Tn*5gusA1* insertions revealed that the genes involved in pantocin B synthesis are located within a 19 to 20 kb region. The direction of transcription in this region was determined to be mostly from right to left, except for a small segment near the left-most portion of the clone, where transcription was in the opposite orientation.

A mini-Tn*5Cm* insertion in pCPP719 and a Tn*5* insertion in pCPP717 were marker-exchanged into Eh318, creating Eh421 (pantocin A-deficient), Eh439 (pantocin B-deficient) and Eh440 (deficient in pantocin A and B). Direct mutagenesis of Eh318 with Tn*5* yielded several colonies that produced reduced inhibition zones in an overlay seeded with *E. amylovora*. No mutant had lost all ability to inhibit *E. amylovora*. Three independent Tn*5* mutants, whose insertions mapped to different portions of the pantocin B biosynthetic region were chosen for further work. The three did not produce pantocin B.

Data from several large immature pear fruit assays with marker-exchange mutants of Eh318 indicated that loss of the ability to produce one or both pantocins reduced protectant ability most of the time. Similar results were obtained in the apple blossom assay in which Eh440 was a significantly poorer protectant than Eh318. Moreover, all three Tn*5* mutants protected immature pear fruit less well than Eh318 but better than the buffer check. The three direct transposon-induced mutants also offered less protection to immature pear fruit than any of the marker-exchange mutants.

Discussion

The regions involved in the synthesis of pantocin A and pantocin B were delineated and the role of the two compounds in biological control of fire blight was assessed. A relatively small region (2.7 kb) and only three genes are involved in pantocin A synthesis. One possible scenario is that the biosynthetic machinery encoded by the three genes utilizes a product that is formed by a housekeeping protein or, alternatively, that pantocin A is made as a prepeptide (much like microcin C7), which is encoded upstream of *paaABC*, which in turn is modified by the products of the *paa* operon to produce pantocin A. Judging by the sequence similarity of the *paaA*-gene product to other proteins in the database, it seems likely that this protein is a biosynthetic enzyme that participates in the formation of pantocin A. The product of *paaC* probably is an integral membrane protein, which may play a role in the export of or resistance to pantocin A. The functional organization of genes in the *paa* operon resembles that of other microcin operons in that the genes whose products are involved in biosynthesis are proceeded by genes whose products are involved in export/resistance (Moreno et al. 1995). The biosynthetic genes of pantocin A are similar in size (2.7 kb vs. 2.4 kb) to those of the antibiotic produced by strain Eh252 of *Pantoea* sp. (Vanneste and Yu 1996).

The biosynthetic region of pantocin B is much larger than that of pantocin A. Its size (19 kb) is similar to that of the antibiotic biosynthetic clusters of several *Pseudomonas* species. For synthesis of pyoluteorin, 21 kb are required (Kraus and Loper 1995), for phaseolotoxin, 28 kb (Zhang et al. 1993), and for syringomycin, ca. 30 kb (Zhang et al. 1995).

In several immature pear fruit assays using marker-exchange mutants of Eh318 deficient in the synthesis of pantocin A and B, only Eh440, the mutant deficient in the production of both antibiotics, consistently performed less well than Eh318. A similar conclusion was reached based on the apple blossom assay using the same mutants. Eh421 and Eh439 did not differ significantly from Eh318 in the ability to control fire blight in blossoms. Based on the results of these two bioassays using marker-exchange mutants, pantocin A and pantocin B together contribute to the biological control efficacy of Eh318. The result of the immature pear fruit assay using the Tn*5* mutants also indicates an important role for pantocin B alone in biological control of fire blight with Eh318.

Other strains of *P. dispersa* or *P. agglomerans* that are effective as biocontrol agents against *E. amylovora* have been subjected to similar analyses. Based on available information, antibiotics seem to play a role in biocontrol in several other cases. For example, Tn*5* insertion mutants of strain Eh252 and Tn*phoA* insertion mutants of strain Eh1087 that are defective in antibiotic production were less effective than wild-type strains in protecting immature pear fruit (Kearns and Mahanty 1993, Vanneste et al. 1992). Whether these transposon insertions have affected structural or regulatory genes or genes involved in antibiotic export is not known. The structural genes of other antibiotics produced by *Pantoea* spp. have been cloned (Davis and Ishimaru 1993, Tenning et al. 1993, Vanneste and Yu 1996), but no sequences have been reported.

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Biological control of phytopathogens by phloroglucinol and hydrolytic enzyme producing bacterial inoculants

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Abstract

Environmental and consumer concerns have focused interest on the development of biological control as an alternative, environmentally-friendly strategy for the protection of agricultural and horticultural crops against phytopathogens. Biological control agents, producing a variety of secondary metabolites and hydrolytic enzymes, have been identified among fungi, actinomycetes and bacteria. *Pseudomonas fluorescens* F113 and *Stenotrophomonas maltophilia* W81 inhibit growth of the phytopathogenic fungus *Pythium ultimum* in vitro and are capable of protecting sugar beet against the effects of damping-off under soil conditions. Transposon mutagenesis of strains F113 and W81 has demonstrated that the biocontrol abilities of these strains are mediated by 2,4-diacetylphloroglucinol (PHL) or lytic enzyme production, respectively. *Globodera rostochiensis* is a phytopathogenic cyst nematode of major agronomic importance. Purified PHL, lytic enzymes, and chitinolytic or phloroglucinol-producing bacterial inoculants negatively influence hatch of *G. rostochiensis* eggs and decrease subsequent viability of juvenile cyst nematodes in vitro. Similar results were obtained under soil conditions.

Introduction

Biological control exploits the negative interactions that occur between disease-suppressive microorganisms and plant pathogens for the improvement of plant health in an environmentally friendly manner (Cook et al. 1995, Handelsman and Stabb 1996). Potential biocontrol agents, mediating plant protection through the production of a variety of hydrolytic enzymes and secondary metabolites (Chernin et al. 1995, Dowling and O'Gara 1994, Howell and Stipanovic 1980, O'Sullivan and O'Gara 1992), have been identified among fungi, actinomycetes and bacteria (Becker and Schwinn 1993, Cook 1993, Dunne et al. 1997a, Keel and Défago 1997). Bacterial strains *Pseudomonas fluorescens* F113 and *Stenotrophomonas maltophilia* W81 were previously isolated from the rhizosphere of field grown sugar beet due to their ability *in vitro* to inhibit growth of the causal agent of damping-off, *Pythium ultimum* (Dunne et al. 1996 and 1997a, Fenton et al. 1992). Transposon mutagenesis of F113 and W81 resulted in the isolation of mutants F113G22 and W81A1 which are unable to antagonize *Pythium ultimum* due to their inability to produce 2,4-diacetylphloroglucinol (PHL) or chitinolytic and proteolytic enzymes, respectively (Cronin et al. 1997a and 1997b, Dunne et al. 1997b, Fenton et al. 1992).

Globodera rostochiensis is a cyst nematode capable of causing extensive damage to potato crops. Traditional protection measures against such damage have included crop rotation and the use of toxic chemical treatments. However, biological control of parasitic nematodes using fungal or bacterial strains has been proposed as an alternative crop protection strategy (Mankau, 1980, Spiegel et al. 1991). Experimental results presented here demonstrate

the importance of PHL and hydrolytic enzyme production in the anti-fungal biocontrol abilities of *P. fluorescens* F113 and *S. maltophilia* W81, and further implicate production of the secondary metabolite PHL in the ability of F113 to negatively influence the life-cycle of *G. rostochiensis* in vitro and in soil. Assessment of the chitinolytic bacterial strains *S. maltophilia* M1-12 and *Chromobacterium* spp. UP1, previously isolated from a soil suppressive to potato cyst nematode infestation (Cronin et al. 1997c), demonstrate the potential of hydrolytic enzyme-producing bacterial inoculants for the control of nematode-mediated disease.

Materials and Methods

Microorganisms and growth conditions. Bacterial strains were routinely grown shaking in Luria Bertani broth (LB) (Sambrook et al. 1989) or Sucrose Asparagine (SA) (Scher and Baker 1982) at 28 °C. *Pythium ultimum* was obtained from the Commonwealth Mycological Institute (Surrey, England) and was maintained on corn meal agar (Difco, Detroit, MI, USA). Cysts of the nematode *Globodera rostochiensis* (virulence group Ro1) were stored dry at 25 °C for four to five months to ensure that the diapause was completed. Prior to performing experiments, the cysts were incubated on filter paper soaking in distilled water for seven days at 20 °C to promote homogenous hatching rates.

Biocontrol efficacy of bacterial inoculants in vitro. In vitro antagonism of *Pythium ultimum* by *P. fluorescens* F113, *S. maltophilia* W81 and their mutant derivatives was determined using the solid medium bioassay previously described by Fenton et al. (1992).

To determine the effects of purified chitinase, protease or collagenase on egg hatch of *G. rostochiensis* in vitro one milliliter volumes of enzyme solution (approximately 0.5 units enzyme/ml) were added to 20 ml of 50 mM sodium phosphate buffer, pH 6.4 in 100 ml Erlenmeyer flasks. Twenty-five cysts were introduced into the flasks and incubated at 12 °C for 8 weeks. At fortnightly intervals six cysts were removed aseptically and placed in 0.3 mM picrolonic acid, an artificial hatching factor, for 28 days. During this period weekly visual counts of hatched eggs per cyst were made and fresh picrolonic acid was added. At the end of the 4 week period the total number of observed eggs hatched was calculated. Lytic enzyme-free buffer was used as a control. The experiment was carried out in triplicate with two groups of three cysts assayed per replicate.

To investigate the ability of chitinolytic or phloroglucinol-producing bacterial inoculants to influence hatch of *G. rostochiensis*, inoculated phosphate buffer containing nematode cysts was incubated at 12 °C for at least 3 weeks, with gentle shaking on alternate days. Cysts were then sampled and exposed to picrolonic acid prior to enumeration of hatched juveniles. Strains tested included wild type *P. fluorescens* F113, PHL-deficient mutant F113G22, *S. maltophilia* M1-12 and *Chromobacterium* UP1. The experiment was performed in duplicate and two groups of three cysts were assayed for egg hatch per replicate.

The influence of *P. fluorescens* F113 and F113G22 on the viability of *G. rostochiensis* juveniles was studied as follows. Approximately 130 nematodes were added to phosphate buffer in the wells of a 96-well titration plate. The plate was incubated at 12 °C for 48 h, with gentle shaking every 12 h, and the ratio of mobile to motionless juveniles determined. Four replicates were studied per treatment.

Biocontrol efficacy of bacterial inoculants in soil. The ability of wild type F113, F113G22, wild type W81 and W81A1 to protect sugar beet against damping-off disease was investigated in natural soil microcosms using procedures previously described by Fenton et al.

(1992). Sugar beet seeds (cv. Accord) were inoculated with approximately 6 log CFU/seed of the bacteria studied. Controls consisted of seeds dipped in quarter-strength Ringer's solution (untreated control) or a solution containing the synthetic fungicides Previcur N (i.e., propamocarb; Schering AG, Germany) at 20 ml/kg seed and thiram at 7.5 g/kg seed (commercial control). Infection of seeds by *Pythium* spp. was assessed daily for the first seven days of the experiment. Plant emergence was determined at 28 days. The experiment was repeated three times.

The influence of bacterial inoculants on cyst nematode egg hatch was evaluated by co-inoculating bacterial inoculants and cysts of *G. rostochiensis* in soil which was then mixed thoroughly. The water content of the soil was maintained at 60% saturation of the soil porosity. The pots were incubated in a growth chamber (12 °C, 16 h photoperiod). Strains tested included wild type F113, mutant F113G22, *S. maltophilia* M1-12 and *Chromobacterium* UP1. Cysts were extracted from the soil as described by Winfield et al. (1987) before incubation in picrolonic acid. Hatched juveniles were counted, as described above. Five replicates were used per treatment and three groups of two cysts were assayed per replicate. The effect of PHL-producing bacterial inoculants on juvenile nematode viability was also evaluated in soil microcosms. The experimental procedures were similar to those described above, except that the soil also contained approximately 200 juveniles per 150 g soil. Juvenile nematodes were reisolated from soil using a Baermann funnel (Van Gundy 1983), counted microscopically and the ratio of mobile to motionless nematodes determined. The experiment was performed in triplicate.

Statistical analysis. Data were analysed by analysis of variance, using procedures of the Statistical Analysis System (SAS Institute, Cary, NC, USA). When appropriate, treatments were compared using Fisher's least significant difference test. All analyses were conducted at $P = 0.05$.

Results and Discussion

Biocontrol of fungi and cyst nematodes by PHL-producing *P. fluorescens* F113. Antagonistic bacteria have received much attention as potential environmentally friendly agents for the control of fungal and nematode pests (Cook et al. 1993, Dunne et al. 1997a). *Pseudomonas fluorescens* F113 is an effective biocontrol agent isolated from the sugar beet rhizosphere and has been the focus of considerable interest due to its ability to antagonize both the fungal pathogen *P. ultimum* and the bacterial potato pathogen *Erwinia carotovora* (Cronin et al. 1997b, Fenton et al. 1992). Previous studies involving transposon mutagenesis of F113 resulted in the isolation of the near-isogenic mutant F113G22 which is incapable of inhibiting growth of *P. ultimum* in vitro due to its inability to produce PHL (Table 1) (Fenton et al. 1992). The development of a high performance liquid chromatography (HPLC) assay allowed investigation of factors influencing secondary metabolite production by F113, and demonstrated that efficient phloroglucinol production by the strain is dependent upon the presence of suitable carbon sources and the availability of iron (Dowling et al. 1996). These results hold major implications for the successful application of microbial biocontrol agents as effective secondary metabolite production may be dependent on both the components of seed and root exudates and the physical parameters of the soil environment (Dowling et al. 1996). Assessment of the influence *P. fluorescens* F113 may exert on the hatching rates and subsequent viability of the cyst nematode *G. rostochiensis* demonstrated that exposure of cysts to F113 increased the ability of *G. rostochiensis* to hatch both in vitro and in soil

microcosms (Table 2). In contrast, the PHL-deficient mutant F113G22 had no effect on hatching rates (Table 2), directly associating PHL production by F113 with its ability to influence cyst nematode hatch. Further evaluation of the effects of wild type F113, and F113G22, on the viability of exposed juvenile nematodes demonstrated that significantly increased mortality occurred only in the presence of the wild type strain (Table 2). As juvenile nematodes of *G. rostochiensis* can persist in soil for a period of only 10 to 12 days in the absence of the host potato plant, a biocontrol strategy exploiting PHL-producing F113 may involve introduction of the microbial inoculant into the soil prior to potato sowing in order to increase hatch. The effects of F113 on nematode juvenile viability may further improve the efficiency of potato plant protection.

Table 1. Evaluation of biological control of *Pythium ultimum* under laboratory and natural soil microcosm conditions.

Treatment	Inhibition of *Pythium ultimum* growth (in vitro)	*Pythium*-free seeds at 24 h in soil (%)	Sugarbeet emergence at 28 days in soil (%)
Commercial control	+	100 a[y,z]	72 (4) a
Untreated control	-	5 (5) b	38 (6) c
F113Rif	+	90 (4) a	59 (4) b
F113G22	-	33 (8) b	41 (4) c
W81	+	87 (9) a	64 (7) ab
W81A1	-	30 (11) b	36 (4) c

[y] Standard deviations are given in parentheses.
[z] Within each column, values with the same letter are not significantly different according to a protected LSD test.

Table 2. Effects of 2,4-diacetylphloroglucinol-producing *P. fluorescens* F113 on hatch and juvenile viability of the potato cyst nematode *Globodera rostochiensis* under in vitro and soil conditions.

Treatment	Untreated control	F113 (10^7 CFU/ml)	F113G22 (10^7 CFU/ml)
In vitro			
Hatched juveniles/cyst	120 (9)[y] a[z]	190 (8) b	111 (7) a
Viable juvenile nematodes/cyst (%)	89 (4) a	28 (3) b	84 (9) a
In soil			
Hatched juveniles/cyst	140 (19) a	229 (78) b	130 (38) a
Viable juvenile nematodes/cyst (%)	85 (18) a	26 (11) b	84 (11) b

[y] Standard deviations are given in parentheses.
[z] Within each row, values with the same letter are not significantly different according to a protected LSD test.

Assessment of biocontrol by hydrolytic enzyme-producing bacterial inoculants. In addition to antibiosis, biological control of both fungal and nematode pests by bacterial strains may be mediated by the production of hydrolytic enzymes (Chernin et al. 1995). Bacterial strains *S. maltophilia* W81, *S. maltophilia* M1-12 and *Chromobacterium* strain UP1, like

P. fluorescens F113, were previously isolated from the soil environment (Cronin et al. 1997c, Dunne et al. 1997a). *S. maltophilia* W81 proved capable of inhibiting growth of *P. ultimum* in vitro and effectively conferred protection on sugar beet seeds against colonization and subsequent damping-off in naturally *Pythium* infested soil microcosms (Table 1). However, a lytic enzyme deficient transposon-induced mutant, W81A1, was unable to antagonize the oomycete under similar conditions (Table 1). Further genetic complementation and biochemical assays have demonstrated that W81 antifungal activity is mediated by the lytic disruption of the structural integrity of the *Pythium* cell wall (*data not shown*). In similar fashion the integrity of the structure of the *G. rostochiensis* egg shell, which is formed from a chitin and protein matrix, can be disrupted by purified commercial hydrolytic enzymes leading to subsequent decreases in egg hatch. However, while chitinase and protease enzymes proved effective when used individually (Table 3) combinations of both hydrolytic enzymes caused significantly greater decreases (Table 3). Evaluation of the chitinolytic bacterial strains *S. maltophilia* M1-12 and *Chromobacterium* strain UP1, under both in vitro and soil conditions, also significantly decreased the ability of *G. rostochiensis* eggs to hatch (Table 3).

Table 3. Effects of purified lytic enzymes and chitinolytic bacteria on hatch of the potato cyst nematode *Globodera rostochiensis*.

Treatment	Egg hatch in vitro (% hatched eggs/cyst)	Egg hatch in soil (% hatched eggs/cyst)
Purified enzymes		
Untreated control	100 a[X]	
Chitinase	43 (6) b[Y]	
Protease	39 (7) bc	
Collagenase	51 (9) b	
Chitinase, protease	26 (7) c	
Chitinase, collagenase	87 (7) a	
Collagenase, protease	58 (6) d	
Chitinase, protease, collagenase	68 (5) d	
Bacterial strains		
Untreated control	74 (11) a	65 (9) a
S. maltophilia M1-12[Z]	29 (7) c	38 (4) b
Chromobacterium spp. UP1[Z]	19 (9) c	40 (5) b

[X] Within each column, values with the same letter are not significantly different according to a protected LSD test

[Y] Standard deviations are given in parentheses.

[Z] Inoculum density for both bacterial strains was 10^Z CFU/ml.

In summary, the results presented here demonstrate the biocontrol efficiency of both hydrolytic enzyme and PHL-producing bacterial inoculants to protect against the effects of phytopathogens. Future strategies may involve evaluation of these biocontrol agents, individually and when used in synergistic combinations, under conditions relevant to current agricultural practices.

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Production of 2,4-diacetylphloroglucinol by a *Pseudomonas* strain in the rhizosphere of maize: implication of this compound in the biological control of *Fusarium graminearum*

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Abstract

Pseudomonas fluorescens strain M.3.1. is an effective antagonist against the maize fungal pathogen, *Fusarium graminearum*. Antagonistic activity is often linked to a production of antibiotic, which is highly influenced by carbon sources. The aim of these experiments was to investigate a M.3.1. production of 2,4-diacetylphloroglucinol in vitro and on soluble exudates of maize, and to correlate this production to its antagonism against *F. graminearum*. After extraction of antibiotic from M.3.1. suspension on KMB, 2,4-diacetylphloroglucinol was detected and purified by TLC. Purified 2,4-diacetylphloroglucinol, as crude extract of antibiotic, reduced the growth of *F. graminearum*. This production of antibiotic was quantified by HPLC analysis, and drastically inhibited the fungal growth under in vitro conditions. Biosynthesis of 2,4-diacetylphloroglucinol was also detected when bacteria was grown on soluble exudates of maize as the only source of carbon. These results demonstrate that M.3.1. is a potential biocontrol agent in situ. Factors influencing the production of antibiotic in situ must be studied in order to better understand the biocontrol activity process of M.3.1.

Introduction

Numerous rhizosphere-inhabiting fluorescent pseudomonads have the ability to promote the plant growth by the colonization of the rhizosphere. They are commonly called PGPR (plant growth-promoting rhizobacteria) (Kloepper and Schroth 1978). They can synthesize phytohormones that directly act on the plant growth (Dowling and O'Gara 1994). They also can produce siderophores and antibiotics that have an undirect action on plant growth by affecting the growth of soilborne fungal pathogen. Bacterial siderophores chelate with a high affinity the soluble iron, which became limiting for the growth of the other rhizophere micro-organisms (Lemanceau 1992). Antibiosis is an important feature implicated in the reduction of the growth of pathogenic microorganisms (O'Sullivan and O'Gara 1992). 2,4-diacetylphloroglucinol is a major antibiotic associated with the supression of pathogenic fungi such as *Thielaviopsis basicola* (Keel et al. 1990) and *Gaeumannomyces graminis* var. *tritici* (Keel et al. 1992). Its precursor, monoacetylphloroglucinol, also has an antibiotic effect. The production of antibiotic is widely influenced by the carbon source. In the rhizosphere, the carbon source is essentially provided by root exudates.

Pseudomonas fluorescens strain M.3.1. was isolated from the rhizosphere of maize. Under gnotobiotic conditions, this strain was an effective antagonist against the pathogenic fungus of maize: *Fusarium graminearum*. Preliminary results have indicated a role of siderophore (pyoverdine) and cyanide in this antagonism (Benizri et al. 1995).

The aims of this study were to investigate a production of monoacetylphloroglucinol and 2,4-diacetylphloroglucinol by *P. fluorescens* strain M.3.1., and to correlate this production with the antagonism of M.3.1. toward *F. graminearum*.The production of antibiotic was quantified by HPLC method in order to evaluate if the bacteria produce enough 2,4-diacetylphloroglucinol to drastically inhibit in vitro the growth of *F. graminearum*. For a better understanding of antibiotic production in situ, antibiotic production was investigated when bacteria was inoculated on soluble exudates of maize as the sole carbon source.

Materials and Methods

Pseudomonas fluorescens strain M.3.1. was isolated by M. Digat (INRA Angers, France) from maize roots grown in Thailand. *Fusarium roseum* var. *graminearum* 51866 was also isolated from maize roots grown in Central Africa, and it is responsible for root rot. Maize cultivar DEA was used (Pionner, France). Seeds were chosen from the model class of 0.26 to 0.28 g.

Fusarium roseum var. *graminearum* was maintained on solid King's B medium (King et al. 1954) at 27 °C in darkness. Maize cultures were conducted hydroponically using the protocol previously described by Benizri et al. (1995). Sterilized seeds were introduced in glass tubes containing Hoagland's solution medium (Hoagland and Arnon 1938). The system was maintained under gnotobiotic conditions during the 3 days of germination and the following 10 days of growth. Solution culture containing soluble exudates was then removed for the inoculation of bacteria. *Pseudomonas fluorescens* strain M.3.1. was inoculated on 50 ml of King's B medium (KMB) or on growth medium of 10 days old maize. All the bacterial cultures were incubated on a rotary shaker (200 rpm) for 120 h at 27 °C in darkness.

To extract antibiotics, bacterial cultures were centrifugated at 3500 rpm for 20 min at 10 °C, and the supernatants were extracted with ethyl acetate (v/v) for 2 h on a rotary shaker. The ethyl acetate extracts were reduced to dryness in vacuo at 35 °C, and the crude bacterial extracts were then dissolved in 1.5 ml of methanol 65% (v/v in water).

Thin Layer Chromatography (TLC) was used to detect 2,4-diacetylphloroglucinol and monoacetylphloroglucinol. Aliquots of the crude bacterial extracts and standard antibiotics were streaked on thin layer plates (Silica gel $60F^{254}$, Merck), and eluted with chloroforme:ethyl acetate:methanol (5:4:1; v:v:v). The plates were then observed under UV light, and bands corresponding to 2,4-diacetylphloroglucinol and monoacetylphloroglucinol were determined by comparison to eluted standard antibiotic.

Fungal inhibition was tested in vitro. Each band of purified 2,4-diacetylphloroglucinol and monoacetylphloroglucinol obtained by TLC separation was removed from the plates and eluted in 1.5 ml of acetone. Acetone eluates were sterilised by filtration (Sartorius, 0.2 µm) and spread onto an half KMB Petri dish. After total evaporation of the acetone, a one-square cm of *F. graminearum* from a 7 days KMB pre-culture was placed at the edge of the antibiotic residue. Plates were then incubated at 27 °C. Similar tests were done with crude bacterial extracts, and pure acetone. Inhibition was observed by prevention of the fungal growth in the direction of the antibiotic residue. The ratio (mycelial growth on antibiotic residue) on (growth of mycelium on KMB) (A/K) was calculated at the third day of culture.

Production of 2,4-diacetylphloroglucinol was quantified using analytical HPLC methods derived from the one described by Keel et al. (1992). Aliquots of filtred crude bacterial extracts (Sartorius, 0.2 µm) were analyzed by a Beckman liquid chromatograph. The reverse-phase column C18 (LiChroCART 250-4, Merck) was thermostatically controlled at 45 °C.

The samples were eluted with a three step gradient of methanol in 0.43% *o*-phosphoric acid from 18% to 23% (0 to 5 min), 23% to 53% (5 to 6 min), and 53% to 61% (6 to 26.8 min). The column was then washed during 10 min with pure methanol, and before each analysis it was conditionned 5 min with the first gradient of elution. The rate flow used was 1 ml/min. The 2,4-diacetylphloroglucinol was detected at a wavelength of 270 nm, its retention time was 22 min. Concentrations of antibiotic were calculated from a standard curve of authentic 2,4-diacetylphloroglucinol.

Results and Discussion

Antibiotics were identified with TLC. With the elution solvents used, authentic 2,4-diacetylphloroglucinol had an R*f* value of 0.59 on TLC plates. A residual quantity of monoacetylphloroglucinol, the biosynthesic precursor of 2,4-diacetylphloroglucinol, could be observed at the R*f* value of 0.4. Both appeared as purple spots. The elution chromatogram of crude bacterial extracts revealed several spots. Compared with R*f* values and color of reference antibiotics, two of them corresponded to 2,4-diacetylphloroglucinol and monoacetylphloroglucinol.

Fungal inhibition was determined in vitro. Ratios A/K of 0.77 and 0.53, respectively, were obtained with 2,4-diacetylphloroglucinol spot and the crude antibiotic extract. As no inhibition was observed with pure acetone, this reduction of growth could directly be linked to antibiotics. The highest ratio with crude bacterial extract could be due to the additive effect of 2,4-diacetylphloroglucinol and monoacetylphloroglucinol, or of another inhibiting product not already identified.

Antibiotic production on KMB was quantified. After 120 h of growth on KMB at 27 °C, 2,4-diacetylphloroglucinol produced by strain M.3.1. and released into the culture supernatant was extracted and quantified. The amount of extracted antibiotic was 126 µg per ml of medium. The antibiotic 2,4-diacetylphloroglucinol inhibited in vitro growth of several *Fusarium* species when added to the medium at concentrations between 10 to 128 µg per ml (Table 1). According to the literature, the 2,4-diacetylphloroglucinol produced on KMB is apparently sufficient to drastically inhibit growth of *Fusarium* spp.

Table 1. Concentrations of 2,4-diacetylphloroglucinol inhibiting 50 or 100% of the mycelial growth.

	Inhibition 50% (µg per ml)	Inhibition 100% (µg per ml)	References
F. o. f.sp. *lycopersici*	16	128	Keel et al. 1992
F. o. f.sp. *ini*	32	128	Keel et al. 1992
F. o. f.sp.*cucumerinum*	10.5[z]	84[z]	Maurhofer et al. 1995

[z] initially as µMol

Antibiotic production was detected when bacteria were grown on maize soluble exudates. Compared to KMB culture, the growth of strain M.3.1. was drastically reduced when it was incubated on 10 days old medium culture of maize (*data not shown*). Nevertheless, a negligeable production of antibiotic was detected. However, the amount of 2,4-diacetylphloroglucinol produced has not yet been determined.

Carbon source is one of the main factors influencing in vitro production of several bacterial metabolites including antibiotics (Shanahan et al. 1992). In the rhizosphere, root exudates are the most important source of carbon. Our results show that strain M.3.1. is able to use soluble maize exudates as only source of carbon to synthesize 2,4-diacetylphloroglucinol.

We demonstrated by TLC that M.3.1. produced in vitro 2,4-diacetylphloroglucinol. This synthesis was directly correlated to the antagonism of M.3.1. against *F. graminearum*. In fact, 2,4-diacetylphloroglucinol produced by strain M.3.1. inhibited in vitro the growth of this fungus. 2,4-diacetylphloroglucinol production was quantified at 126 µg/ml by HPLC analysis. According to the literature, this amount was sufficient to drastically inhibit in vitro the development of various *Fusarium*. M.3.1. used maize root exudates as the only source of carbon to synthesize 2,4-diacetylphloroglucinol. Then, we conclude that this bacterial strain is in situ a potential biological agent. Our perspectives are to study some factors modulating in field the root exudation of maize (temperature, mineral nutrition), and to investigate consequences of exsudation variations on the production of 2,4-diacetylphloroglucinol. Moreover, it appears essential to quantify production of antibiotic per bacteria since culture media compositions are more or less favorable to organisms growth, and secondary metabolite synthesis is not necessarily correlated with growth rate.

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Biocontrol of fungal root diseases by 2,4-diacetylphloroglucinol-producing pseudomonads with different restriction profiles of amplified 16S rDNA

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Abstract

Fluorescent pseudomonads producing the antimicrobial 2,4-diacetylphloroglucinol (Phl) are widespread in certain soil and are being studied extensively for use as biocontrol agents of soil-borne fungal diseases. Some of them can produce pyoluteorin (Plt) in addition to Phl, whereas others synthesise only Phl. In the current work, a collection of seven Phl-plus Plt-minus pseudomonads, seven Phl-plus Plt-plus pseudomonads and seven Phl-minus Plt-minus biocontrol pseudomonads was used to investigate the relationship between biocontrol traits *in vitro* and protection of plants against fungal pathogens in vivo. The seven Phl-plus Plt-plus pseudomonads were identical by restriction analysis of amplified spacer ribosomal DNA (spacer ARDRA), whereas the Phl-plus Plt-minus pseudomonads and especially the Phl-minus Plt-minus biocontrol pseudomonads were quite diverse by spacer ARDRA. Collectively, the Phl-plus Plt-minus pseudomonads proved superior to the Phl-plus Plt-plus pseudomonads and the Phl-minus Plt-minus biocontrol pseudomonads for protection of tomato against Fusarium crown and root rot (in rockwool microcosms) or cucumber against Pythium damping-off (in non-sterile soil microcosms). There was no correlation between protection in vivo and inhibition of the corresponding fungal pathogen on plates. However, there was a significant correlation between the amount of Phl produced on plates and protection of tomato against Fusarium crown and root rot, but not with protection of cucumber against Pythium damping-off. No link was found between disease suppression *in vivo* and the amount of Plt synthesised on plates. Overall, results suggest that promising biocontrol pseudomonads may be identified based on the ability to produce Phl or specific ARDRA-based fingerprints.

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Analysis of a gene cluster involved in the biosynthesis of a new salicylic acid-based siderophore and its implication for the suppression of Fusarium wilt by *Pseudomonas fluorescens* WCS374

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Abstract

Siderophore production by fluorescent *Pseudomonas* spp. has been demonstrated to be involved in control of soilborne plant pathogens by these bacteria. It has been suggested that both competition for iron and induction of systemic resistance are the effective mechanisms of siderophore-mediated disease suppression. *Pseudomonas fluorescens* WCS374 induces resistance in radish against *Fusarium oxysporum* f.sp. *raphani* and iron-regulated metabolites were demonstrated to play an important role. The present study revealed that WCS374 produces pseudobactin, salicylic acid (SA), and a novel SA-based siderophore, fluorebactin, under conditions of iron limitation. A region of the WCS374 genome containing the loci necessary for SA and fluorebactin production was cloned and partly sequenced. Together with the sequence data, preliminary mass spectrometric data suggest that fluorebactin contains both SA and histamine moieties. Using reverse transcriptase-PCR it was demonstrated that expression of fluorebactin biosynthesis genes is iron-regulated.

Introduction

Under iron-limited conditions bacteria produce a large variety of low molecular weight ironchelating compounds called siderophores (Neilands 1981). Several strains of fluorescent *Pseudomonas* spp. can produce more than one type of siderophore, enabling the bacteria to effectively compete for iron and reduce the activity of plant-pathogenic micro-organisms (Buysens et al. 1996). Observed bacteria-mediated plant disease suppression not only results from growth restriction of the pathogen by means of sequestering iron from the environment (O'Sullivan and O'Gara 1992), but also from induction of systemic resistance (Duijff et al. 1993, Leeman et al. 1996, Maurhofer et al. 1994). Salicylic acid (SA) is also produced by *Pseudomonas* strains under iron-limiting conditions (Ankenbauer and Cox 1988, Anthoni et al. 1995, Leeman et al. 1996, Meyer et al. 1992, Visca et al. 1993). SA has been described as an inducer of systemic resistance in plants (De Meyer and Höfte 1997, Vernooij et al. 1994) as well as a precursor or intermediate in the biosynthesis of microbial siderophores (Anthoni et al. 1995, Carmi et al. 1994, Cox et al. 1981).

Pseudomonas fluorescens WCS374 increases radish yield through reduction of Fusarium wilt under commercial greenhouse conditions. The main mechanism in this disease suppression appears to be induction of systemic resistance (Leeman et al. 1995a), and lipopolysaccharides (LPS) were suggested to play a major role (Leeman et al. 1995b).

However, at low iron availability, an LPS mutant of WCS374 still protected radish against *Fusarium oxysporum* f.sp. *raphani* to the same extent as the wild type strain, suggesting that iron-regulated metabolites were involved in induced systemic resistance (ISR) (Leeman et al. 1996). It was demonstrated that pseudobactin can trigger ISR in radish. Moreover, under iron-limiting conditions, WCS374 produced SA. Leeman et al. (1996) further suggested that WCS374 may produce additional siderophores that are involved in ISR. In the present study we identified and characterized the SA biosynthesis region of strain WCS374. Evidence was obtained that SA biosynthesis in WCS374 is linked to the synthesis of a novel siderophore.

Materials and Methods

Bacterial strains used were *P. fluorescens* WCS374 (Geels and Schippers 1983), *P. putida* WCS358 and its pseudobactin mutant JM218 (Marugg et al. 1985), *E. coli* S17-1 (Simon et al. 1983), DH5α and HB101 (Boyer and Roulland-Dussoix 1969). SA production from culture supernatants was measured according to the method described by Meyer et al. (1992) and Leeman et al. (1996). Standard procedures were used for DNA electrophoresis, DNA transfer from agarose gels to Nylon membranes, preparation of competent cells and transformation (Sambrook et al. 1989). Isolation of recombinant plasmids was perforemd by alkaline lysis (Birnboim and Doly 1979). Purification of plasmid DNA and elution of DNA restriction fragments were performed using the Qiagen (Qiagen Inc.) systems, according to the instructions provided. DNA restrictions and ligations were performed with enzymes from Pharmacia, according to the manufacturer's instructions. Hybridization was done using the chmiluminescence method with the nonradioactive detection kit from Boehringer Mannheim Biochemicals. DNA sequencing was performed using an Applied Biosystems Model 373A automated DNA sequencer. Sequence analysis and homology searches were performed with the Genepro version 4.0 (Riverside Scientific Enterprises) and the BLAST programs (Altschul et al. 1990) NCBI network service. The nucleotide sequence of the *P. fluorescens* WCS374 SA and fluorebactin biosynthetic region has been deposited in the EMBL, GeneBank and DDBJ nucleotide sequence databases under accession region has been deposited in the EMBL, GeneBank and DDBJ nucleotide sequence databases under accession number Y09356. Reverse transcriptase (RT)-PCR was performed on total RNA (RNeasy^{tm} kit from Qiagen), obtained from WCS374 grown in standard succinate medium (SSM) with and without 100 μM of $FeCl_3 \times 6\,H_2O$. RNA samples were treated with RNase-free DNase (Pharmacia Biotech) to avoid contamination with DNA traces. cDNA synthesis was carried out with the First-Strand cDNA Synthesis Kit (Pharmacia Biotech.), using primer SAL03. PCR reactions were performed with primer pairs DBH0a/DBH02 and SAL01/SAL02. Suppression of Fusarium wilt of radish by *P. fluorescens* WCS374 and its mutant derivatives was studied in a soil bioassay, as previously described (Raaijmakers et al. 1995).

Results and Discussion

The screening of more than 4000 Km-resistant Tn5 insertion mutants of strain WCS374 allowed the isolation of several non-fluorescent mutants. However, all mutants showed siderophore activity on CAS medium agar plates (Schwyn and Neilands 1987). with no significant differences in halo size upon incubation at two different temperatures: 28 °C, optimal for the growth of the strains, and 37 °C, that is repressive for pseudobactin production (Marugg et al. 1985). All putative pseudobactin-minus mutants produced detectable amounts

of SA, although in significantly lower quantities than WCS374. Finally, all mutants displayed a blue fluorescence in SSM medium under UV irradiation. One mutant (374-05) was isolated that showed an enhanced pseudobactin production, a reduction in halo size at 37 °C, and absence of SA production in SSM medium. A second insertion of a modified version of transposon Tn5 (Hynes et al. 1989) in mutant 374-05, aimed to obtain pseudobactin-minus and SA-minus mutants (i.e., mutant 374-05.1), demonstrated that double Tn5 derivative mutants had lost the typical blue fluorescence previously detected and showed reduced halos on CAS medium agar plates. These results indicated that strain WCS374 produced a second siderophore at low iron availability, and led us to hypothesize that production of SA and production of the second siderophore in strain WCS374 are related. Known SA-based siderophores were not previously detected in spent medium of WCS374 and pseudobactin-minus mutants. However, from the culture supernatant of a pseudobactin-minus mutant (374-08) a compound with siderophore activity was isolated and designated fluorebactin. Preliminary mass spectrometry data obtained from fluorebactin, reveal both SA and histamine moieties in the molecule. The structure of fluorebactin is now being further analyzed.

In order to localize the biosynthetic region of SA, a gene bank of WCS374 was mobilized into the pseudobactin mutant JM218 of *P. putida* WCS358, and transconjugants were screened for siderophore activity. If fluorebactin and SA production are linked in strain WCS374, halo restoration due to expression of fluorebactin genes in JM218 would mark SA biosynthesis. Hence, positive clones were screened for SA production. After the screening of some 2.150 transconjugants one clone (pmB374-07, 28 Kb insert size) was identified that conferred not only production of SA, but also of fluorebactin.

An internal 5-kb *Eco*RI fragment from this clone appeared to be fully responsible for SA production (Fig. 1A). Sequence analysis of this 5-kb fragment revealed the presence of four open reading frames (ORF). Two ORFs (*fbsC* and *fbsB*, from fluorebactin synthesis) showed homologies with chorismate-utilizing enzymes. FbsC has homology with isochorismate synthases of different microorganisms. Isochorismate synthase activity is involved in the biosynthesis of siderophores, such as enterobactin, pyochelin, and amonabactin. FbsB showed significant homology with PchB of *P. aeruginosa* (Serino et al. 1995), involved in SA biosynthesis in this bacterium. FbsE showed strong similarity with enzymes involved in siderophore biosynthesis (i.e., enterobactin, pyochelin, and yersiniabactin). The region also contains a putative histidine decarboxylase gene (*fbsA*), which catalyzes the decarboxylation of histidine to histamine.

In vivo expression of the *fbs* operon in *E. coli* revealed that deletions affecting *fbsB* abolished the production of SA, indicating that this gene is essential for SA biosynthesis. Based on the sequence analysis we designet specific primers to study *fbs* gene expression using the RT-PCR technique (Fig. 1B). SAL03 was used for cDNA synthesis and primer pairs SAL01/SAL02 (within *fbsB*, 214 bp PCR expected product) and DBH01/DBH02 (partially overlapping the *fbsCE* genes, 439 bp PCR expected product) were used for subsequent amplification in the PCR reaction. The two primer pairs were designed to produce both predicted PCR products only if the template cDNA used in the reactions was obtained from a ploycistronic mRNA. Repressions of *fbs* gene expression by iron was also studied. Possible contaminations of DNA in the RNA interfere in studying gene expression using RT-PCR. Therefore, the total extracted RNA was treated with RNase-free DNase. To check the effectivity of the DNase treatment, total DNA (2 µg) of WCS374 was also treated with RNase-free DNase and subjected to PCR using both primer sets. RNA from mutant 374-08 was used as a control since it contains both *fbs* genes and the *neo* gene from Tn5 which is

being expressed in the presence of kanamycin. cDNA synthesis of *neo* mRNA was obtained using primer KM03. The primer couple KM01/KM02 was used for subsequent amplification (405 bp PCR expected product). RT-PCR products of WCS374 cultures grown under high and low-iron conditions are shown ind Fig. 1B. The expected PCR products were obtained with both pairs of primers for cDNA obtained from WCS374 grown under low iron conditions. Under high iron conditions a strong reduction of transcript was observed. A semi-quantitative determination of *fbs* transcript, based on dilution series of cDNA, indicated a 100-fold repression by iron (*data not shown*). PCR control treatments of WCS374 genomic DNA treated with RNase-free DNase did not yield any product, indicating that PCR bands were not due to DNA contamination in RNA samples. From these experiments it can be concluded that the *fbsCEAB* genes form a single transcriptional unit and that expression of the genes is iron-regulated. Currently, we are investigating possibilities to use the RT-PCR technique for studying gene expression in the rhizosphere.

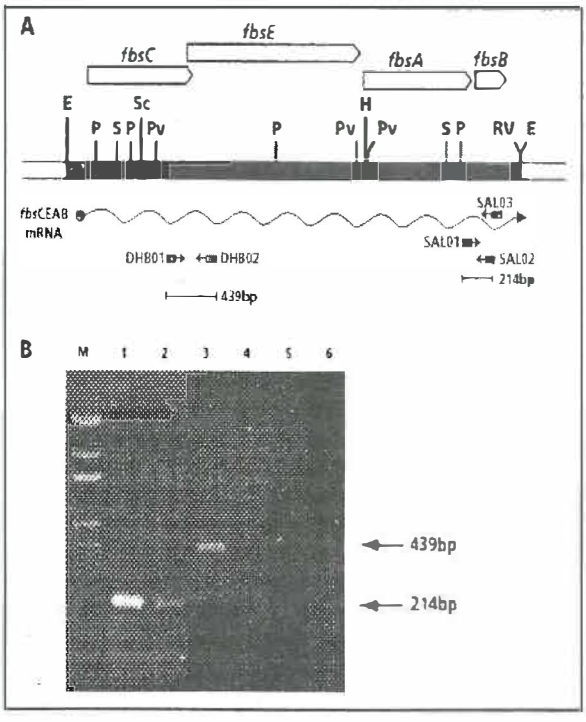

Fig. 1. (A) Physical and genetic organization of the *fbs* region and strategy designed to study expression of the *fbsCEAB* genes in *P. fluorescens* WCS374 and their repression by iron. The position of primers and lengths of expected PCR products are indicates underneath the solid bar. Primer SAL03 was used for cDNA synthesis. (B) RT-PCR products of bacterial cultures grown under low (LI) and high iron (HI) conditions. The primers used are indicated in brackets []. Lanes 1: WCS374 (LI) and 2: WCS374 (HI) [SAL01/ SAL02]; 3: WCS374 (LI) and 4: WCS374 (HI) [DHB01/ DHAB02]; 5: WCS374 DNA treated with RNase-free DNase [SAL01/ SAL02], and 6: WCS374 DNA treated with RNase-free DNase [DHB01/ DHB02].

Suppression of Fusarium wilt of radish by WCS374 and siderophore-deficient mutants was demonstrated in pot bioassays (Table 1). All strains tested significantly reduced disease incidence. Interestingly, mutant 374-08 (pseudobactin-minus) was even more effective in disease suppression. Decrease in SA production in this mutant is correlated with an enhanced production of fluorebactin (*data not shown*). We speculate that enhancement of fluorebactin production, probably due to pseudobactin deficiency, might result in a better biocontrol

activity of this mutant derivative. The involvement of fluorebactin in ISR against *F. oxysporum* f.sp. *raphani* in radish by WCS374 will be further studied using selected mutants of this strain and also purified fluorebactin.

Table 1. Suppression of Fusarium wilt in radish by *Pseudomonas fluorescens* WCS374 and its derivatives.

Treatment	Percentage of diseased plants[z]		
	I	II	III
Control	66.2 a	71.1 a	51.1 a
WCS374	24.4 b	58.9 ab	35.6 b
374-05	28.9 b	44.4 bc	28.0 b
374-05.1	26.7 b	43.6 bc	13.3 c
374-08	15.6 c	38.9 c	14.4 c

[z] In each column percentages with a different letter are significantly different ($P = 0.05$)

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Similarities and differences in the mode-of-action of two rhizosphere bacteria antagonistic to *Globodera pallida* on potato

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Abstract

The two rhizosphere bacteria *Bacillus sphaericus* strain B43 and *Agrobacterium radiobacter* G12, originally isolated from the potato rhizosphere, significantly reduced *Globodera pallida* infestation on potato (*Solanum tuberosum* cv. Hansa). Combined application of B43 and G12 improved overall control consistency. Both antagonistic strains mainly reduced penetration and establishment of nematode juveniles in the root system. Juveniles which formed a syncytium developed normally and no differences in sex ratio or number of eggs/cyst were detected. Both strains were tested in a split-root system for ability to induce systemic resistance against *G. pallida.* Induced resistance was triggered by vital and heat-killed bacterial cells of both strains and in the case of B43also by bacterial culture filtrates. Current progress made in our group on understanding the mode-of-action of these nematode-antagonistic rhizosphere bacteria is reviewed. The results indicate strain-specific mechanisms of nematode control, including induced systemic resistance and bacterial metabolites.

Introduction

Besides controlling plant diseases caused by viruses, bacteria, and fungi, recent studies also indicated control potential of rhizosphere bacteria against plant-parasitic nematodes (Hasky-Günther 1996, Martínez-Ochoa et al. 1997). The two rhizosphere bacteria *Agrobacterium radiobacter* strain G12 and *Bacillus sphaericus* strain B43 are antagonistic to the nematode *Globodera pallida* on potato. Both bacteria were tested positive to induce systemic resistance (ISR) (Hasky-Günther 1996). Nematode control potential ranged from 20% in the field (Racke and Sikora 1992) up to 60% under greenhouse conditions (Hoffmann-Hergarten et al. 1997), regardless soil water status and temperature (Hackenberg and Sikora 1994). The effect of both bacterial strains on nematode life-cycle and their possible mode-of-actions were studied in detail indicating that the two bacteria have some characteristics in common but differ in others (Hasky-Günther 1996). The objectives of this presentation are to (i) review the work of our group on G12 and B43 over the past 10 years and (ii) to summarize the similarities and differences in the mode-of-action of these two antagonistic bacteria.

Materials and Methods

B. sphaericus was cultivated in tryptic soy broth (pH 7.0) and *A. radiobacter* G12 in King's Medium B (pH 5.8). Washed bacterial cells (OD_{560nm}= 2.0) of approx. log 10 CFU/ml were applied as a soil drench to one side of the split-root system. The other side of the root system was inoculated 24 h later with cysts of *G. pallida* (approx. 1,500 eggs plus juveniles/pot). Plants were evaluated for juvenile penetration 5 days after inoculation (DAI), sex ratio

26 DAI, and multiplication 35 DAI (Reitz, *personal communication*). In a search for the inducing agent, vital and heat-killed bacterial cells as well as bacterial culture filtrates were applied to the root system (Hasky-Günther 1996). For the biochemical studies, potato plants were grown in heat-treated sand and inoculated with B43 and G12 three weeks after planting. Samples were taken 1, 3, 5, 7 and 12 days after bacterial treatment. Plant protein studies included SDS-PAGE, Western blot and mRNA hybridization techniques (Hasky-Günther 1996). For the immunological characterization of protein bands the proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then treated with specific polyclonal antibodies raised against common PR-proteins including PR-14, acidic and basic chitinases, acidic and basic β-1,3-glucanases, and PAL. In an additional approach to further characterize key proteins the presence of mRNA inducing chitinase and glucanase expression was evaluated using cDNA probes for basic and acidic chitinases and glucanases, respectively. Polyclonal antibodies and cDNA probes for the above mentioned detection of PR-proteins were provided by the Max-Planck Institute for Plant Breeding, Cologne, Germany.

Comparison between G12 and B43. Application of bacterial strains G12 and B43 to one side of a split-root system significantly reduced *G. pallida* penetration on the other side of the root by 55% and 58%, respectively (Hasky-Günther 1996). Although the overall control level was similar for both bacterial strains, they acted differently as summarized in Table 1.

Table 1. Similarities and differences of the two rhizosphere bacteria *Agrobacterium radiobacter* strain G12 and *Bacillus sphaericus* strain B43 antagonistic to *Globodera pallida* on potato

Similarities	Differences
Plant response - both bacteria induced systemic resistance in the split-root system - bacteria-mediated ISR was caused by vital and heat-killed bacterial cells - systemic resistance was induced by applying 2 ml bacterial suspension to the plant 10 days after planting - no ISR response following foliar application of bacteria - no plant-growth promotion - no expression of common PR-proteins (acidic/basic chitinase, ß-1,3-glucanase, PR-14, PAL)	**Plant response** - culture filtrates of B43 induced resistance, but culture filtrates of G12 did not - G12 induced expression of a novel protein (38kD) - root exudates supported growth of G12 and B43; however, addition of sucrose mainly favored B43 and the resulting fermentation product was toxic to *G. pallida* juveniles - root exudates of G12-treated plants showed relative increase of C-16 and C-18 fatty acids; B43-treated plants did not
Effect on *Globodera pallida* life cycle - increased larval attraction towards the roots - reduced juvenile penetration into the roots - no effect on larval development - no effect on sex ratio - no effect on number of eggs/cyst	**Effect on *Globodera pallida* life cycle** - no significant differences between the bacteria
Bacterial characteristics - both bacteria showed pectolytic and cellulytic activity on indicator media; no proteolytic, lipolytic and chitinolytic activity was detected	**Bacterial characteristics** - while B43 mainly colonizes the rhizosphere, G12 is a predominant colonizer of the rhizoplane and also colonizes root tissue, where it stays locally in the root cortex - Culture filtrates of B43 completely killed *G. pallida* juveniles within 18 hours; culture filtrates of G12 only paralyzed the nematode and juveniles could be reactivated by transferring them into water

Discussion

The main effect of bacteria-mediated induced resistance in our system was a reduction in juvenile penetration. Juveniles which still established in the root system developed normally. At the molecular level application of G12 was associated with the appearance of a novel protein band (38 kd), which could not be recognized as one of the common PR-proteins. However, the importance of this newly expressed protein in the disease complex will be further investigated. For B43-treated plants no differences in protein expression were observed when compared with untreated plants. Although the degree of control was similar for both strains their mode-of-action might be different. The two antagonistic bacteria colonize different ecological niches of the plant-soil environment. While B43 colonizes predominantly the rhizosphere, G12 also colonizes the root internally. Nevertheless, G12 does not spread systemically within the plant, so that spatial separation of bacterium and nematode in the split-root system was still guaranteed. The potential role of endophytic colonization for control efficacy is still unknown and object of current studies. In summary, antagonistic bacteria have some potential to reduce damage caused by plant-parasitic nematodes. Although intensive work was conducted over the last years to study bacteria-nematode interactions, the mode-of-action still needs further investigations.

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Mechanisms involved in the antifungal activity of the rhizobacterium *Serratia plymuthica*

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Abstract

Sixteen strains of *Serratia plymuthica* were isolated from the rhizosphere of oilseed rape. All of the isolates showed an antifungal activity against the phytopathogenic fungus *Verticillium dahliae* var. *longisporum* in vitro. The efficacy of strains varied. The antifungal mechanisms of the selected strains were investigated. Direct antifungal activity may be based on production of the antibiotics prodigiosin and pyrrolnitrin, and the lytic enzymes chitinase and ß-1,3-glucanase. Potent siderophores were secreted by most of the strains to improve the availability of iron. None of the strains produced hydrogen cyanide. The mechanisms were specific for each isolate. Chitinolytic activity is exclusively responsible for the antifungal activity of *S. plymuthica* C48 in vitro. The chitinolytic enzymes were estimated by using assays with chromogenic *p*-nitrophenyl analogs of disaccharide, trisaccharide, and tetrasaccharide derivates of *N*-acetylglucosamine. Additionally, fluorescent substrates with a 4-methylumbelliferyl group linked by ß-1,4 linkage to *N*-acetylglucosamine mono- or oligosaccharide were used to identify the chitinolytic activity of proteins. Three enzymes were detected: one *N*-acetyl-ß-D-glucosaminidase, one chitobiosidase and an endochitinase. Mutants with decreased chitinolytic activitiy were obtained after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment. While the wild-type C48 showed inhibition zones up to 20 mm in dual culture against *V. dahliae* var. *longisporum*, some mutants had no inhibitory activity. Mutant *S. plymuthica* C48*chi*^{minus}3/4 was unable to protect plants of oilseed rape against Verticillium wilt.

Introduction

Members of the genus *Serratia* have antifungal activity and have frequently been reported to be associated with plants. For example, *S. plymuthica* was found as a common rhizobacterium with antifungal activity against *Fusarium culmorum* and *Pythium* spp. (Alström and Gerhardson 1988). Bacterial antagonism responsible for the benefit of plants and biological control, may be due to the production of antibiotics, competition, or parasitism (Chet et al. 1990). Some strains of *S. plymuthica* produce a non-diffusible red pigment, called prodigiosin, which has antifungal activity. Another antibiotic, pyrrolnitrin, was found in strains of *S. plymuthica* (Kalbe et al. 1996). Parasitism relies on lytic enzymes for the degradation of cell walls of pathogenic fungi. Microorganisms capable of lysing other organisms can serve as a powerful tool for biological control (Chet et al. 1990). Many rhizobacteria with chitinolytic activity have been described, including *Aeromonas caviae* (Inbar and Chet 1991), *S. marcescens* (Ordentlich et al. 1988), and *Enterobacter agglomerans* (Chernin et al. 1997). Induced systemic resistance, broadly defined as activation of latent defense mechanisms in plants prior to pathogenic attack, has been hypothesized in recent years to be an operable mechanism in several rhizobacterial systems (Kloepper et al. 1992). It is also known, that *S. plymuthica* is able to induce systemic resistance to *Colletotrichum orbiculare* (Kloepper et al. 1992). The objective of this study was to characterize the antifungal mechanisms of *S. plymuthica* isolated from the rhizosphere of oilseed rape.

Materials and Methods

The strains of *S. plymuthica* used in this study were isolated from the rhizosphere of oilseed rape (*Brassica napus* L. ssp. *oleifera* Metzg. Sinsk) at different locations in Mecklenburg-Hither Pomerania (Kalbe et al. 1996). *Serratia* strains were grown at 30 °C in nutrient broth (Sifin, Berlin, Germany) or on peptone glycerol agar (PGA; 5 g Bacto-Peptone (Difco, Detroit, MI), 10 ml Gycerol, 20 g agar per liter water). *Verticillium dahliae* var. *longisporum,* isolated from oilseed rape in 1987, was from the culture collection of the University of Rostock, Microbiology.

The ability of the bacteria to inhibit growth of *V. dahliae* var. *longisporum* was determined in a paired in vitro assay on Waksman agar as previously described (Berg 1996).

To detect prodigiosin, bacteria were cultivated on PGA at 30 °C for 48 h. Cells were harvested and dissolved in 25 ml acetone for 30 min. The solution was centrifugated at 10,000 *g* for 10 min. The supernatant was then purified by gel filtration on sephadex LH-20 (Pharmacia, Uppsala, Sweden) using methanol:distilled water (1:1 vol/vol) as eluting solvent. The active fractions were concentrated under vacuum (50 °C) and then the dry residue was dissolved in 1 ml of ethanol. The spectrum of the red pigment was compared with prodigiosin according to Süssmuth et al. (1987). For detection of pyrrolnitrin, bacteria were cultivated at 30 °C for 72h on nutrient agar (NA; Gibco) amended with 2% glycerol. The purification of pyrrolnitrin was carried out according to Kraus and Loper (1992). The purified extracts of both antibiotics were tested in a bioautography assay (Kalbe et al. 1996).

Detection of chitinolytic activity was done on plates. Bacterial colonies were screened for chitinase production and excretion by plating on chitin agar (CA; 1.62 g nutrient broth (Sifin), 0.5 g NaCl, 6 g M9 salts, 2 g chitin, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 3 nM Thiamin-HCl (all from Sigma), 15 g agar per liter distilled water). Clear halos indicating enzymatic degradation were measured after 5 days of incubation at 30 °C. Chitinolytic activity was also determined in liquid culture supernatants. Enzymatic activity was measured by using assays with chromogenic *p*-nitrophenol (*p*-NP) analogs of di-, tri- and tetrasaccharids of *N*-acetylglucosamin (basic compound of chitin) in culture filtrate according to Chernin et al. (1995). The release of the chromophore *p*-NP from the substrates was measured at 410 nm, and 1 U of enzymatic activity was defined as 1 µmol of *p*-NP per mg protein per hour. Chitinolytic enzymes were detected after gel electrophoresis as follows. Proteins were concentrated 20 fold in AMICON Ultrafiltration Cell System (AMICON INC. Beverly, USA) through a membrane of 10 kDa. The proteins were separated by native and sodium docecyl sulfate (SDS)-7.5% polyacrylamide gel electrophoresis (PAGE). Enzymes were detected on gels using fluorescent substrates described by Tronsmo and Harman (1993). The chitinolytic enzymes appeared as fluorescent bands under UV light because of enzymatic hydrolysis of the fluorescent substance 4-methylumbelliferone from the *N*-acetylglucosamine mono- and oligosaccharides. The following substances were used: 4-Methylumbelliferyl-*N*-Acetyl-β-D-Glucosaminide (4-MU-GlcNAc), 4-Methylumbelliferyl-β-D-*N,N'*-Diacetylchitobioside [4-MU-$(GlcNAc)_2$], 4-Methylumbelliferyl-β-D-*N, N', N''*-Triacetylchitotriose [4-MU-$(GlcNAc)_3$] (all from Sigma). β-1,3-glucanase activity was determined by measuring the production of reducing sugars from laminarin (Sigma) according to Daugrois et al. (1990).

To analyze the ability of selected bacterial isolates to produce siderophores we used the plate assay according to Schwyn and Neilands (1987). Hydrogen cyanide production was determined Aquaquant 14417-Testsystem (Merck) using liquid cultures of the isolates.

NTG-mutagenesis was conducted as follows. Bacterial cells of *S. plymuthica* C48 were suspended at $1.5x10^7$ CFU/ml in 10, 40, 100, 200 and 400 ppm of *N*-methyl-*N'*-nitro-*N*-

nitrosoguanidine (NTG) (Sigma) and incubated for 10, 20, 30, 40, and 60 min at 30 °C. Mutants were obtained from single colonies grown on the plate of PGA in which about 1% of bacteria survived. Mutants were screened in the fungal inhibition assay. For further tests, 2505 mutants from four wild-type isolates obtained were selected after mutagenesis.

Greenhouse experiments were conducted as follows. Soil samples (compost, peat, field soil 1:1:2, vol.) were infested with *V. dahliae* var. *longisporum* (10 g microsclerotia/kg soil). Polypropylene boxes (7 x 7 x 8 cm) were filled with infested soils and rape seeds (*Brassica napus* "Lambada"). Bacteria were applicated solved in distilled water (1 ml 10^8 cells). The experiments were conducted under greenhouse conditions at 20 to 33 °C with daily irrigation. The disease index was determined according to Zeise (1992).

Results and Discussion

The isolates were screened for their ability to suppress *V. dahliae* var. *longisporum* in an in vitro bioassay (Table 1). The efficacy of the isolates was different. We have tried to prove the involvement of antifungal mechanisms by the antagonistic bacteria. We found only three strains that produced prodigiosin (Table 1). Most isolates produced pyrrolnitrin. Only three strains (isolate GP 1, GP2, P184) showed an activity of β-1,3-glucanase. For half of the isolates chitinolytic activity could be detected (Table 1). Most of the isolates of *Serratia* (75%) produced siderophores. No isolate produced cyanide. The antifungal mechanisms of rhizosphere bacteria include production of antibiotics (antibiosis), production of siderophores (competition), production of lytic enzymes like chitinase and beta-1,3-glucanase and production of cyanide (induction of resistance in the host plant). We investigated the production of siderophores, enzymes, cyanide and the antibiotics prodigiosin and pyrrolnitrin. It is necessary to prove the mechanisms for each isolate, because the production and especially the quantity of secondary metabolites are often specific for a strain. This could also be confirmed for our isolates (Table 1). Six different combinations of antifungal mechanisms were found in the sixteen isolates. The combination of antibiotics, siderophores and lytic enzymes were found for three isolates (GP1, GP2, GP38). Most of the isolates possess two antifungal mechanisms antibiotics combined with siderophores (C2, C3, C53, C54, P485) or antibiotics combined with lytic enzymes (C46, C50, P184). This supports the thesis of Fravel (1988), that antibiosis is involved in most antagonism and is often combined with other mechanisms. The cooperation of siderophores and lytic enzymes were found in 2 isolates (C44, C47) and only one mechanism was found in C45, C52 (siderophores) and C48 (chitinases). Two different antibiotics were isolated, prodigiosin and pyrrolnitrin. Only two strains produced the red pigment and antibiotic prodigiosin. Pigmented *Serratia* strains were rarely isolated from plants (Grimmont and Grimmont 1992). Strains producing the pigment prodigiosin seem to be toxic to protozoa and this may be an ecological advantage in water and soil (Grimmont and Grimmont 1992) and perhaps in the rhizosphere.

Table 1. Antifungal mechanisms of *Serratia plymuthica*[z]

Isolate	Prodigiosin	Pyrrolnitrin	Siderophores	Glucanase	Chitinase	HCN
GP1	-	+	+++	+	+	-
GP2	-	+	+++	+	+	-
C2	+	+	++	-	-	-
C3	+	+	++	-	-	-
C38	-	+	+++	-	+	-
C44	-	-	+++	-	+	-
C45	-	-	+++	-	-	-
C46	-	+	-	-	+	-
C47	-	-	++	-	+	-
C48	-	-	-	-	+	-
C50	-	+	-	-	+	-
C52	-	-	+++	-	-	-
C53	-	+	+	-	-	-
C54	-	+	+	-	-	-
P184	-	+	-	+	-	-
P485	-	+	+++	-	-	-

[z] Antibiotic activity of prodigiosin and pyrrolnitrin: '+' or '-' indicates antifungal activity in bioautograph or lack of, respectively.
Siderophores detected according to Schwyn and Neilands (1987): '+++' represents 20 mm orange zone, '++' represents 5-20 mm orange zone, '+' represents 5-3 mm orange zone.
β-1,3-glucanase detected following Daugrois et al. (1990): '+++' indicates > 10 U per ml, '++' indicates 1-10 U per ml, '+' indicates 0.1-1 U per ml.
Chitinase detected on plates: '+' represents hydrolysis, '-' represents no hydrolysis.
Production of hydrogen cyanide (HCN) determined with Merck Schnelltest 14417: '-' represents < 0.001 mg/liter.

Chitinolytic activity of *S. plymuthica* C48 was determined for the wild-type and mutants. The wild-type hydrolyzed colloidal chitin after 72 h of grown on semiminimal agar supplemented with colloidal chitin as the sole carbon source. Large zones of clearing around the growing bacteria were observed. Some mutants were unable to form clearing zones. With this plate assay only chitinolytic activity could detected and this gave no indication of quality of chitinases. It was qualitatively determined using assays with chromogenic *p*-nitrophenyl analogs of disaccharide, trisaccharide, and tetrasaccharide derivates of *N*-acetylglucosamine. The strain C48 showed activity in extra- and intracellular proteins in assays with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-β-D-*N, N'*-diacetylchitobiose and *p*-nitrophenyl-β-D-*N, N', N''*-triacetylchitotriose (Table 2). The chitinases were induced when the strain was grown in the presence of colloidal chitin as the sole carbon source. Chitinolytic activity was significant lower for mutant C48*chi*^{minus}3/4 (Table 2). Additionally, the activity was determined by using three fluorescent substrates with a 4-methylumbelliferyl group linked by β-1,4 linkage to *N*-acetylglucosamine mono- or oligosaccharids after separation by gel electrophoresis. Three enzymes were detected on native gels: a *N*-acetyl-β-D-glucosaminidase using exclusively 4-MU-(GlcNac), a chitobiosidase hydrolyzes only 4-MU-$(GlcNac)_2$ and an endochitinase with activity on substrates 4-MU-(GlcNac), 4-MU-$(GlcNac)_2$ and 4-MU-$(GlcNac)_3$. Reduced chitinolytic activity could be detected by the mutant. These results indicate that mutant is deficient in chitinases production.

Table 2. Chitinolytic activity of extracellular proteins of *Serratia plymuthica* C48 and chitinolytic negative mutant *S. plymuthica* C48*chi*^{minus}3/4 activity grown with chitin as the sole carbon source

	Time (h)			
	24	48	72	96
***Serratia plymuthica* C48**				
p-nitrophenyl-N-acetyl-β-D-glucosaminide	5.41	4.18	25.64	31.28
p-nitrophenyl-β-D-N,N'-diacetylchitobiose	1.43	1.87	13.74	17.59
p-nitrophenyl-β-D-N,N',N''-triacetylchitotriose	2.50	0.91	4.47	7.95
***S. plymuthica* C48*chi*^{minus}3/4**				
p-nitrophenyl-N-acetyl-b-D-glucosaminide	0.43	0.10	1.09	1.29
p-nitrophenyl-β-D-N,N'-diacetylchitobiose	0.39	0.42	0.82	1.19
p-nitrophenyl-β-D-N,N',N''-triacetylchitotriose	0.98	1.32	1.18	1.80

The involvement of chitinases in biocontrol of strain *S. plymuthica* C48 was studied by using *V. dahliae* var. *longisporum* in oilseed rape as a model, comparing wild-type with the mutant C48*chi*^{minus}3/4 with reduced chitinolytic activity. When *S. plymuthica* C48 were applied as a bacterial suspension mixed with the seed, the number of plants with symptoms of Verticillium wilt decreased (16% disease reduction). When mutant which lost the chitinolytic activity, was used no significant reduction was observed. This demonstrates the importance of *S. plymuthica* chitinases in biological control. The results presented in this work support previous findings demonstrating a role for chitinases with other microbial antagonists, including *A. caviae* (Inbar and Chet 1991), *S. marcescens* (Ordentlich et al. 1988), and *E. agglomerans* (Chernin et al. 1995, 1997).

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A promising new metabolite from *Pseudomonas fluorescens* for biocontrol of *Pythium ultimum* and *Rhizoctonia solani*

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Abstract

Screening for *Pseudomonas* strains active in biocontrol of *Pythium ultimum* and *Rhizoctonia solani* resulted in the isolation of *P. fluorescens* DR54, which showed antagonistic activity both in vitro and in planta. Antagonistic activity was also observed when extracts from spent growth media of *P. fluorescens* DR54 were tested against the two pathogenic fungi. Fractionation of the extract by semi-preparative HPLC resulted in isolation of an active compound, which resembled the lipopeptide, viscosin. ^{1}H-NMR and HPLC analysis indicated, however, that the compound had a unique structure, which is currently being characterized. In liquid media the metabolite was extractable from both the supernatant and cell fraction, but most activity was found in the cell fraction. The active compound was produced when DR54 was cultured in both liquid and solid media, and in both complex and defined media with various carbon sources. Metabolite production under a variety of growth conditions is a promising feature of strain DR54 in relation to biocontrol under in situ conditions.

Introduction

In vitro screening for *Pseudomonas fluorescens,* antagonistic against the two root pathogenic fungi, *Pythium ultimum* and *Rhizoctonia solani,* resulted in isolation of several active isolates (M. Nielsen, J. Sørensen, J. Fels, and H. Pedersen, *unpublished data*). However, only a few of the isolates showed a stable antagonism on several different media, which is considered useful for prediction of in situ antagonistic activity on seeds, where the exploitable carbon sources may be complex. Pot experiments confirmed that the active isolates all had good antagonistic effect on plants (M. Nielsen, J. Sørensen, J. Fels, and H. Pedersen, *unpublished data*).

One of the isolates, *P. fluorescens* DR54, was found to produce a new antagonistic compound, viscosinamide, which is active against the pathogenic fungi, *P. ultimum* and *R. solani.* In this paper we show that viscosinamide production by *P. fluorescens* DR54 takes place in both complex and defined media, using different carbon sources.

Materials and Methods

Pseudomonas fluorescens DR54 was inoculated to a density of 0.1 and grown at 28 °C in a shaking liquid culture (120 rpm) for 2 days in complex liquid media: Potato Dextrose Broth (PDB, Difco), and Luria Broth (LB, 1% Bacto tryptone, 0.5% yeast extract, 1% NaCl and 0.01% glucose, pH 7.2). As defined liquid media, we used Davis Minimal Broth (DMB, Kragelund and Nybroe 1994) without Na-citrate, but with different single carbon sources to a final concentration of 50 mM. *Pseudomonas fluorescens* DR54 was inoculated to a density of

0.1 using washed cells from an overnight culture in DMB with glucose and grown at 28 °C in a shaking culture for 2 days at 120 rpm. *Pseudomonas fluorescens* DR54 was streaked out and incubated at 26 to 28 °C for 4 to 7 days on Potato Agar (PA), Potato Dextrose Agar (PDA, Difco) and Seed Extract Agar (SEA, M. Nielsen, J. Sørensen, J. Fels, and H. Pedersen, *unpublished data*).

Cells were removed from the liquid culture by centrifugation at 7,000 *g* for 10 min at 4 °C. The cell fraction was extracted with acetone, which was subsequently evaporated. Cells and extracted material were suspended in methanol before filtration (0.2 µm) and HPLC analysis of the filtrate. Supernatant and total culture liquids were extracted twice using 1.5 volume of ethyl acetate, with 0.1 % formic acid in the first extraction. After evaporation of the ethyl acetate, the extract was dissolved in methanol, filtered (0.2 µm) and analyzed on HPLC.

Analytical detection of viscosinamide with HPLC was achieved using a LiChroCART 250-4 HPLC-Cartridge with LiChrosphere 100 RP-18 (5 µm) column, kept at 40 °C and acetonitrile/0.1% *o*-phosphoric acid gradient from 80/20 to 100/0 (1 ml/min) over a 20 min period, measuring absorption at 210 nm.

Results

The new active compound viscosinamide is related to viscosin isolated from *P. viscosa* (Kochi et al. 1951) and *P. fluorescens* (Hildebrand 1989). Viscosinamide production was observed when *P. fluorescens* DR54 was cultured on both PA, PDA, SEA, PDB and LB (*results not shown*). In a study with PDB as the substrate, *P. fluorescens* DR54 showed an initial exponential increase in OD with no viscosinamide production, followed by an linear growth phase which initiated a large increase in viscosinamide production (Fig. 1A). During the linear growth period the viscosinamide production reached a nearly constant level per OD_{600} unit (Fig. 1B).

Among the defined media, we illustrate *P. fluorescens* DR54 viscosinamide production during a growth experiment on sucrose. Growing on sucrose, *P. fluorescens* DR54 exhibited a slow exponential increase in viscosinamide during a 3 day period, with most of the viscosinamide present in the cell fraction and a low and nearly constant amount in the supernatant (Fig. 2A). It was also observed that the viscosinamide production seemed to follow the changes in optical density (i.e., the cell number) (Fig. 2A) resulting in a nearly constant high amount of viscosinamide per OD_{600} unit (Fig. 2B).

In other defined media (Fig. 3) total extracts also showed high viscosinamide production by *P. fluorescens* DR54 in sucrose and mannitol media (Fig. 3A) while glutamate, glucose, and asparagine showed low production per ml. The viscosinamide production per cell was less in mannitol, sucrose, glutamate, glucose, and asparagine amended media (Fig 3B). Viscosinamide was produced on all media, albeit with different levels per unit of cell mass.

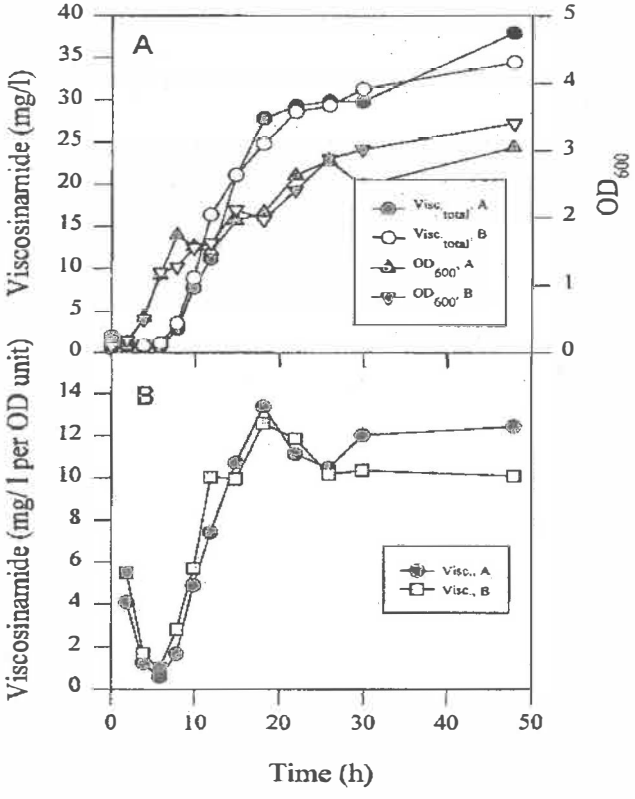

Fig. 1. Viscosinamide production by *P. fluorescens* DR54 in potato dextrose broth.

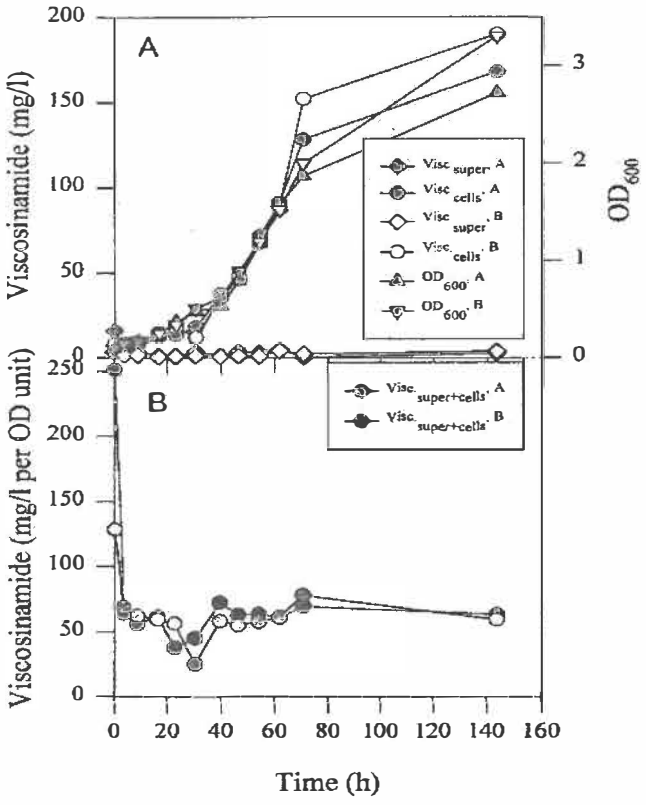

Fig. 2. Viscosinamide production by *P. fluorescens* DR54 in DMB media with sucrose.

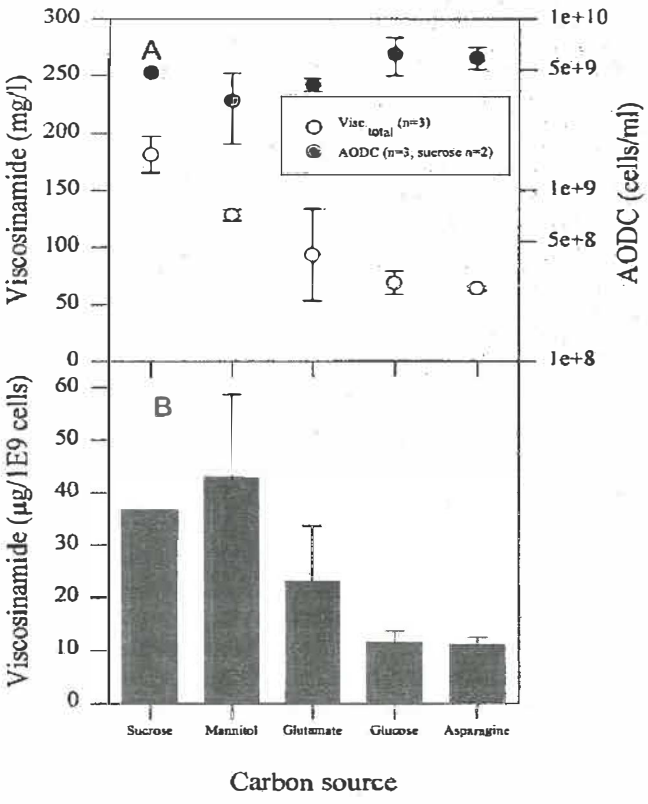

Fig. 3. Viscosinamide production by *P. fluorescens* DR 54 in DMB media with different carbon sources.

Discussion

Isolate *P. fluorescens* DR54 produced the depsipeptide viscosinamide which is structurally similar to a group of compounds resembling viscosin. Viscosinamide production was detected in different complex media, both in liquid and solid forms, and representing rich (PDA, PDB, and LB) and low nutrient composition (SEA and PA) (M. Nielsen, J. Sørensen, J. Fels, and H. Pedersen, *unpublished data*).

In PDB medium the amount of viscosinamide varied during the incubation period (Fig. 1) with no production during initial growth phase, followed by an increase to a constant level during subsequent growth. The initial lack of production in PDB was not seen in experiments with defined carbon sources and may be caused by an unknown repressor in the complex PDB medium.

In defined medium with sucrose as carbon source, the amount of viscosinamide per OD unit was high and constant. Similar results were obtained for the production of viscosinamide during growth on other defined carbon sources allthough the amount of viscosinamide

produced per cell depended on the growth substrate. The low but nearly constant amount of viscosinamide in the supernatant suggested that *P. fluorescens* DR54 may regulate the excretion of viscosinamide to the surrounding media. The viscosinamide production on all tested media suggested that *P. fluorescens* DR54 may be a good candidate for biological control of pathogenic fungi on seeds, where complex and variable carbon sources may be present.

The direct effect of viscosinamide on *P. ultimum* and *R. solani* has been investigated by Thrane et al. (1998) using vital fluorescent probes for staining the fungal hyphae. In both pathogenic fungi, viscosinamide promotes cellular changes including extended branching, hyphal swelling, frequent septation, and changes of esterase activity, pH, and mitochondrial activity in the hyphae. It is, however, still unknown whether viscosinamide production is causing the antagonistic effect observed in plant experiments (M. Nielsen, J. Sørensen, J. Fels, and H. Pedersen *unpublished data*).

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Interactions between *Stenotrophomonas maltophilia* and the soilborne pathogen *Verticillium dahliae* var. *longisporum*

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Abstract

Strains of *Stenotrophomonas maltophilia* were isolated from the rhizosphere of oilseed rape. Eleven isolates showed antifungal activity against *Verticillium dahliae* var. *longisporum* in vitro but the level of antagonism varied among strains. Several mechanisms of action appeared to be involved, including production of antibiotics, lytic enzymes, and iron-chelating siderophores. Production of the antibiotic maltophilin appeared to be a primary mechanism of antifungal activity for isolate R3089, based on comparisons of the wild-type and a less antagonistic, maltophilin negative Tn5 derivative, R3089::Tn5-1.

Introduction

Stenotrophomonas maltophilia (Hugh) Palleroni and Bradbury (syn. *Pseudomonas maltophilia* and *Xanthomonas maltophilia*, Palleroni and Bradbury 1993) is a ubiquitous bacterium which is often associated in high numbers with plants, particularly crucifers. Crucifers produce high levels of sulphur-containing compounds including methionine (Berg et al. 1996, Debette and Blondeau 1980) which is required by *S. maltophilia* (Ikemoto et al. 1980). *Stenotrophomonas maltophilia* has been suggested to have properties useful in the biological control of soilborne plant pathogens (Elad et al. 1987, Lambert et al. 1987). *Stenotrophomonas maltophilia* isolate R3089 reduced the incidence of Verticillium wilt in oilseed rape by about 23%, increased yield about 9%, and increased thousand seed weight by 10% in a field trial (Berg et al. 1994). *Stenotrophomonas maltophilia* is also thought to play an important role in element cycling in nature (Ikemoto et al. 1980).

In this study we investigated the antifungal activity and mechanisms of action of *S. maltophilia* against *Verticillium dahliae* var. *longisporum* Stark, an important soilborne pathogen which causes tracheomycosis and wilt of oilseed rape (*Brassica napus* L. ssp. *oleifera* Metzg. Sinsk). Oilseed rape has become an increasingly important crop in Europe. Over the years very short rotations have led to a build-up of Verticillium wilt followed by yield losses.

Materials and Methods

Strains of *S. maltophilia* were isolated from the rhizosphere of oilseed rape at different locations in Mecklenburg-Hither Pomerania (Berg et al. 1996). *Stenotrophomonas* strains were grown at 30 °C in nutrient broth (Sifin, Berlin, Germany) or in GYCA medium (0.5% yeast extract, 0.5% glucose, 4% $CaCO_3$, 1.5% agar). *Escherichia coli* S17-1 (pSUP5011) carrying Tn5-mob and resistance to ampicillin, chloramphenicol, and kanamycin (Simon et al. 1983) was grown at 37 °C in LB broth (Difco Laboratories, Detroit, USA). Antibiotics were

used when appropriate with (μg/ml): ampicillin (50), kanamycin (50), and imipenem (2.5). *Verticillium dahliae* var. *longisporum* isolated from oilseed rape in 1987 was obtained from the culture collection of the University of Rostock, Microbiology.

The ability of the bacteria to inhibit growth of *V. dahliae* var. *longisporum* was determinated by the paired in vitro assay on Waksman agar described by Berg (1996).

To determine production of maltophilin and other antifungal compounds, *S. maltophilia* was grown for 2 days in 300 ml nutrient broth at 30 °C with shaking at 120 rpm. Fermentation broth was centrifuged at 10,000 *g* for 10 min. and the supernatant was passed through a SERVACHROM XAD-2 column (Serva, Heidelberg, Germany). After washing with two volumes of distilled water and one volume acetone-water (1 : 1) the active substances were desorbed with one volume methanol. The eluate was concentrated under vacuum and further purified by gel filtration on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) using ethanol as the eluting solvent. The active fractions were concentrated under vacuum and the dry residue was dissolved in 1 ml of ethanol. The eluate was analyzed using thin-layer chromatography on silica gel plates (Merck) with butanol:ethyl-acetate:distilled water (3 : 1 : 1 vol/vol/vol) for development and the presence of maltophilin was determined with HPLC as described by Jacobi et al. (1996). The presence of antifungal compounds was determined by bioautography. The thin-layer chromatogram was first autoclaved, then overlayed with a thin layer of Czapek-Dox agar (GIBCO, Paisley, Scotland), and a conidia suspension of *Verticillium*, and incubated at 20 °C for 5 days. Siderophore production was assayed on plates according to Schwyn and Neilands (1987). Chitinase production and excretion was assayed on Chitin Agar (CA; 0.162% nutrient broth, 0.05% NaCl, 0.6% M9 salts, 0.2% chitin, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 3 nM Thiamin-HCl, and 1.5% agar). Clearing of the agar around bacterial colonies indicated chitinase production. β-1,3-glucanase activity was determined by measuring the production of reducing sugars from laminarin (Sigma) according to Daugrois et al. (1990). The standard assay (1 ml) contains the enzyme extract, 2.5 mg laminarin, 100 mM acetate buffer pH 5.2. The laminarin substrate was dissolved in acetate buffer by heating at 60 °C before use. The reaction mixture was incubated 1 h at 50 °C. Total reducing sugars were assayed by colorimetric method and expressed as maltose equivalents.

The role of maltophilin in antifungal activity was determined using Tn5 mutagenesis. Biparental matings were conducted with *E. coli* S17-1 as the donor carrying Tn5 on the suicide plasmid pSUP5011 and *S. maltophilia* R3089 as the recipient. Logarithmically growing cultures of S17-1 (5 h) and R3089 (20 h) were grown with appropriate antibiotics. Donor and recipient were concentrated at 10^7 cells/ml with centrifugation, pellets were resuspended in 1 ml LB broth, and incubated together in spots on nutrient agar for 12 to 16 h at 30 °C. Bacteria were harvested by suspending in 3 ml PBS (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) and incubated on kanamycin amended GYCA for 48 to 72 h at 30 °C. Antibiotic resistant transconjugants were screened for fungal inhibition in vitro (Berg 1996). Putative antifungal-negative mutants were identified on the basis of a lack of inhibition compared with wild type R3089.

Results

Eleven isolates of *S. maltophilia* were screened for their ability to suppress *V. dahliae* var. *longisporum* in vitro. Three isolates showed a strong effect in vitro with an inhibition zone about 10 mm (Table 1). Most isolates showed a middle inhibition against the pathogen, but all isolates had an antifungal effect. We have tried to demonstrate the involvement of different

mechanisms in pathogen inhibition. Bioautography of culture filtrates demonstrated production of antifungal compounds by isolates R3089, P30, P32 and P35 (Table 1). All isolates produced different levels of siderophores on a plate assay. Isolates R3089, P27, P30, P42, and P74 produced β-1,3-glucanase while only isolate R3089 produced chitinase.

The role of maltophilin in fungal inhibition was analyzed with Tn5 mutagenesis. Putative transconjugants derived from random Tn5 insertions into the genome of *S. maltophilia* were screened for loss of in vitro activity against *V. dahliae* var. *longisporum*. Mutant R3089::Tn5-1 had reduced antifungal activity. The wild-type showed activity in a bioautography assay but the mutant did not. The mutant deficient in maltophilin production but was not deficient in production of siderophores nor β-1,3-glucanase and chitinase.

Table 1. Antifungal mechanisms of *Stenotrophomonas maltophilia*[z]

Isolate	Fungal inhibition	Antibiosis	Siderophore	Glucanase	Chitinase
R3089	+++	yes	+	++	+
P27	+	no	++	+++	-
P30	++	yes	++	+	-
P32	++	yes	++	-	-
P35	++	yes	++	-	-
P37	++	no	++	-	-
P41	++	no	++	-	-
P42	++	no	+++	+	-
P44	++	no	+	+	-
P74	+++	no	+	-	-

[z] Fungal inhibition determined in dual culture with *V. dahliae* var. *longisporum*; '+++' inhibition zone > 10 mm, '++' inhibition zone 5 to 10 mm, '+' inhibition zone 0 to 5 mm. Antibiosis determined with bioautography after thin-layer chromatography of culure filtrates. Siderophores produced on agar according to Schwyn and Neilands (1987); '+++' orange zone 20 mm, '++' orange zone 5 to 20 mm, '+' orange zone 5 to 3 mm. Beta-1,3-glucanase activity determined following Daugrois et al. (1990); '+++' indicates > 10 U/ml, '++' indicates 1 to 10 U/ml, '+' indicates 0.1 to 1 U/ml. Chitinase activity determined on plates; '+' indicates hydrolysis of chitin, '-' indicates no hydrolysis.

Discussion

The in vitro antifungal activity against *V. dahliae* var. *longisporum* varied among *S. maltophilia* isolates. The antifungal effect of isolates *S. maltophilia* is a due to a combination of several mechanisms. Direct antifungal activity may be based on the production of siderophores by all isolates. *Stenotrophomonas maltophilia* not only produce their own siderophores, but are able to utilize iron-complexes of the pseudobactin siderophores from other bacteria as an iron source (Jurkevich et al. 1992). Production of lytic enzymes such as chitinase and glucanase may also contribute to the antifungal activity of some strains. Some of the isolates produced an antifungal compound identified as the macrocyclic β-lactam antibiotic, maltophilin (Jacobi et al. 1996). Additionally isolates of *S. maltophilia* were able to produce plant growth hormones including indol-3-acetic acid (Berg and Ballin 1994).

The results of this study indicate that antibiosis may be a primary mechanism for the inhibition of *V. dahliae* var. *longisporum* by *S. maltophilia* R3089. A transposon mutant of *S. maltophilia,* R3089::Tn5-1, was deficient in maltophilin production and had reduced in vitro antifungal activity. Production of antibiotics has been described as a major factor in disease suppression by antifungal rhizobacteria (Fravel 1988).

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Establishment in tomato plants and mechanism of action of fluorescent pseudomonads antagonistic to the Fusarium wilt pathogen

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Abstract

Antagonistic effects of different isolates of fluorescent pseudomonads were tested in vitro and in vivo against Fusarium wilt of tomato. Roots of 3-week-old tomato plants were cut and dipped in bacterial suspension with a concentration of $2x10^9$ CFU/ml for 30 min. Six days later the roots of tomato plants were recut and dipped in conidial suspension of *Fusarium oxysporum* f.sp *lycopersici* with a concentration of $5x10^6$ spores/ml. Isolates W34, W21, WB15 and WB52 showed antagonistic effects in vitro and in vivo against *F. oxysporum* f.sp *lycopersici*. The fluorescent pseudomonads were reisolated from tomato stems after one, three and seven days in a concentration of $1x10^4$ CFU/g fresh weight and after two and three weeks in a concentration of $1x10^2$ CFU/g. The concentration of salicylic acid in King's B (KB) broth was determined after 42 h of incubation. The isolates tested differed in production of salicylic acid. WB52 and W34 also produced cyanide in high quantity after three days on KB agar. Antifungal substances were extracted from isolates WB52 and W34 and detected by bioautographic method using *F. oxysporum* f.sp *lycopersici* and *Cladosporium* sp. as test organisms.

Introduction

Availability of bioagents for the commercial application in comparison to pesticides is not yet satisfactory (Schwars 1992), particularly against soilborne diseases, due to the following reasons: (i) establishment of the bioagents in the soil is not stable, because they are affected by negative abiotic and biotic factors and hence the effectiveness of these bioagents against the soilborne pathogens is not sufficient, however, in vitro a lot of antagonistic bioagents effectively control a number of pathogens (Baker et al. 1982), (ii) methods of application need some precautions and improvement in efficiencies, (iii) specificity of the bioagents, and (iv) formulation difficulties. Fusarium wilt of tomato caused by *Fusarium oxysporum* f.sp *lycopersici* W.C. Snyder and H.N. Hans has a drastic effect on yield in greenhouses as well as in open fields, irrespective of the planting seasons and the growth stages. Fluorescent pseudomonads are involved in the natural suppressiveness of some soils to different plant pathogens. These bacteria have also been applied successfully to suppress Fusarium wilts of various plants (Lemanceau and Alabouvette 1993). This study was concentrated mainly on the establishment of fluorescent pseudomonads in the xylem of tomato plants and the possible mechanism of action against *F. oxysporum* f.sp. *lycopersici*.

Materials and Methods

In vitro, the antagonistic effects of 73 isolates of fluorescent pseudomonads were tested on King's medium B (KB) against *F. oxysporum* f.sp. *lycopersici*. Bacterial isolates (24 h-old-culture) were streaked on the middle of KB plates (with five replications for each one) and incubated at 26 °C. Four days later, malt agar plugs of *Fusarium* (2 week-old-culture) were

placed 5 mm away from the edge of the bacterial streak and the plates were incubated at 26 °C for one week. In vivo studies, 35 isolates of fluorescent pseudomonads were tested against Fusarium wilt of tomato in the greenhouse. The roots of 3-week-old tomato plants were cut and dipped in bacterial suspension with a concentration of 2 x 10^9 CFU/ml for 30 min. After 6 days, the roots of these plants taken out of the pots gently, were recut and dipped in conidial suspension of *F. oxysporum* f.sp. *lycopersici* with a concentration of 5 x 10^6 spores/ml and replanted in the same pots. One month later, the foliar symptom index and the vascular browing index were determined on a 0 to 3 and 0 to 4 scale, respectively. The production of salicylic acid from the effective bacterial isolates was tested in KB-broth after 42 h of incubation. After the centrifugation of bacterial cultures at 2,600 *g* for 20 min, 1.0 ml of the supernatant of each sample acidified to pH 2.0, was extracted with 2.0 ml of chloroform. The chloroform phase was taken to dryness with a flow of nitrogen, and the residue was resuspended in 1.0 ml distilled water. Salicylic acid (SA) was quantified with HPLC equipped with a fluorescence detector set at 304 and 408 nm excitation and emission, respectively (Press et al. 1997). To study the production of cyanide (HCN) from the effective bacterial isolates, the isolates (24 h-old-culture) were streaked on KB plates. Sterilized filter papers dipped in alkaline sodium picrate solution (5g picric acid; 25g Na_2CO_3; 2 liter H_2O) were placed inside the lid of the petri dishes and sealed with parafilm. These plates were observed daily for the color changing from yellow to reddish brown due to the absorption of HCN by the picrate solution (Millar and Higgins 1970). To extract antifungal substances from the effective isolates, fluorescent pseudomonads were cultured on semisolid minimal sucrose asparagine medium (0.35% agar) and incubated at 28 °C. The entire contents of 10 plates of each isolate were placed in a 1 liter flask adding 250 ml of 80% aqueous acetone following the several processes as described by Shanahan et al. (1992). Yellow residues were obtained and streaked on thin layer chromatography plates (Kieselgel 60 F254, Merck®) and developed in tert-butylmethylether-hexan-methanol (50:40:3.5 vol/vol). The bands were viewed under long and short wave UV light to see the fluoresces and absorbtion. The antifungal effects of the bands were detected by bioautographic method using *F. oxysporum* f.sp. *lycopersici* (1x10^6 spores/ml) or *Cladosporium* sp. (1.2x10^7 spores/ml) as test organisms.

Results and Discussion

Four isolates, W34, W21, WB15 and WB52 showed antagonistic effects in vitro as well as in vivo against *F. oxysporum* f.sp. *lycopersici*. In vitro, the isolates W34, WB15 and WB52 varied in their actions from fungistatic to fungicidal depending on the age of the fungal and bacterial cultures, time interval between bacterial inoculation and placing of fungus plugs on the agar plates, and the kind of medium used. The fluorescent pseudomonads could be reisolated from tomato stems after 1, 3, and 7 days of inoculation in a concentration of 1x10^4 CFU/g fresh weight and even after 2 and 3 weeks in a concentration of 1x10^2 CFU/g, which demonstrated that fluorescent pseudomonads can survive in the xylem of tomato plants for a long period. However, in comparsion to drenching method, the bacterial isolates could not be reisolated from stem tissue of different plants. Similar results have also been reported by van Peer et al. (1990). The concentrations of salicylic acid obtained in KB-broth are presented in Table 1. It is well known that the quantity of SA (2-hydroxybenzoic acid) plays a very important role in the reduction of disease severity and induction of systemic resistance in plants against foliar diseases (White 1979, Yalpani et al. 1991). Press et al. (1997) showed that SA produced by *Serratia marcescens* 90-166, is needed for induction of systemic

resistance with this strain, but SA is not the only bacterial determinant involved.

Table 1. Production of salicylic acid (SA) by some fluorescent *Pseudomonas* isolates after 42 h incubation in King's B-broth.

Isolate	SA (µg/L)
WB15	5.74
W34	21.88
WB52	12.56
W21	32.11
G54	0
G307	0
G309	0

However, in the present study, some other factors besides SA appear to be involved in determining the effectiveness of these bioagents in controlling the diseases. Isolates WB52, W34 and G309 produced cyanide in a high concentration after 3 days of incubation (Table 2). The concentration of HCN is related to the intensity of color changing from yellow to reddish brown. Défago et al. (1990) reported that there is a positive correlation between the production of HCN and enhancement of the growth of hairy roots as well as the antagonistic effect. However, HCN production is negatively correlated with the disease severity and incidence. Several antifungal compounds appear to be produced by W34 and WB52 based on bioautography of culture filtrates using *Cladosporium* spp. as the test organism (Fig. 1).

Table 2. Production of cyanide in vitro by some of the fluorescent *Pseudomonas* isolates on King's medium B and Pseudomonas agar P.

Isolate	King's B[z]	PAP[z]
WB52	3	3
G307	3	6
W34	4	4
G308	7	5
G309	7	5

[z] Time interval (days) from bacterial inoculation until changing the color of the filters through cyanide production.

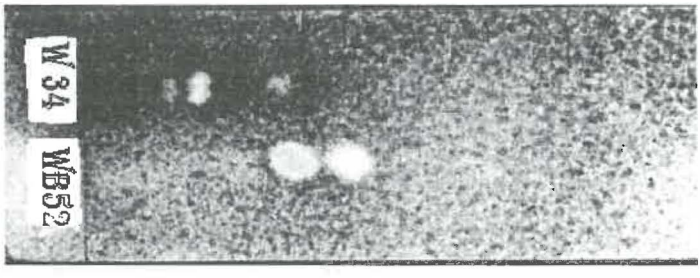

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Fig. 1. Production of antifungal substances by W34 and WB52 detected by thin layer chromatography and bioautographic method using *Cladosporium* spp. as a test organism.

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Detection of fungal stress responses by vital fluorescent staining

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Abstract

Vital fluorescent staining is a promising tool for detection of physiological changes induced by antifungal compounds. We have used fluorescent stains successfully to indicate the occurrence of a physiological response in the plant pathogenic fungi *Rhizoctonia solani* and *Pythium ultimum* when exposed to viscosinamide, a new lipopeptide metabolite purified from the antagonistic bacterium *Pseudomonas fluorescens* strain DR54. Extension of the study to other soil fungi supported the general applicability of vital fluorescent staining in studies of stress-affected intracellular structures and activities.

Introduction

There has been a tremendous increase in the availability of vital fluorescent stains with high specificity to different cell structures as well as stains suitable as indicators of specific cell physiological states (Haugland 1996). Among the advantages with using such stains, are the low cellular toxicity, high intracellular stability, and the better reflection of spatial and temporal variability than other microscopy techniques.

Most experience with vital fluorescent staining are found in cell biology studies of animal cells. In studies related to fungal physiology or ecology, however, until recently only two vital fluorochromes habe commonly been used. One is FDA (fluorescein diacetate) that fluoresces after cleavage by intracellular esterases and has been used as a general indicator of metabolic activity. The other is calcofluor white that stains glucan and chitin structures of the cell walls. The applicability of these stains in dynamic studies over time is limited as the FDA-staining has very little stability in light and calcofluor white requires UV-excitation which is harmful to fungi.

In the field of biological control of fungal pathogens, little is known about the physiological effects caused by secondary metabolites produced by bacterial antagonists. In an initial study we have focused on developing methods for monitoring cellular changes in soil fungi induced by a new antifungal compound (C. Thrane, T.H. Nielsen, J. Sørensen, and S. Olsson, *unpublished data*). Among a number of vital stains tested, six were selected on the basis of staining quality and their ability to detect fast, intermediate, and slow cellular stress reactions. In the present report are shown examples of staining of intracellular structures and metabolic activity in unstressed hyphae of the Oomycete *Pythium oligandrum* Drechsler, the Zygomycete *Rhizopus arrhizus* Fischer, and the Basidiomycetes *Antrodia vaillantii* De Candolle, and *Hypholoma fasciculare* (Huds.: Fr) Kummer. Finally, an example of a stress response of *Rhizopus arrhizus* to a crude extract of metabolites from *P. fluorescens* DR54 is shown.

Materials and Methods

Fungi. Mycoparasitic *P. oligandrum* isolate 1010, *R. arrhizus*, *A. vaillantii*, and *H. fasciculare* were used in this study.

Antifungal compounds. A crude extract of extracellular metabolites of the antagonistic *P. fluorescens* DR54 according to T.H. Nielsen, C. Christophersen, U. Anthoni, and J. Sørensen (*unpublished data*) was used as a crude source of viscosinamide. This extract contained viscosinamide and a range of other compounds as studied by HPLC-analysis (T.H. Nielsen, C. Christophersen, U. Anthoni, and J. Sørensen, *unpublished data*). The extract was applied to small filters that were subsequently placed on the plates inoculated with the fungus.

Assay. The fungi were grown on agar plates containing GAsnM according to Olsson (1995) and were incubated at 20 °C. Vital fluorescence staining of the fungi was carried out directly on the plates according to C. Thrane, T.H. Nielsen, J. Sørensen, and S. Olsson, (*unpublished data*). The specimens were studied in a Zeiss epifluorescence microscope.

Results and Discussion

The use of four different vital stains in four different fungi is illustrated in Fig. 1. The nuclei of *P. oligandrum* were 1 to 2 µm in a diameter and were evenly distributed in the coenocytic hyphae (Fig. 1A). In *R. arrhizus* both membranes, walls and lipid bodies were visible by blue light excitation (Fig. 1B). The lipid bodies were small or absent in some fungi and in others only membranes or walls were stained by this dye (C. Thrane, T.H. Nielsen, J. Sørensen, and S. Olsson, *unpublished data*). Mitochondria of *A. vaillantii* (3 to 6 µm) were longer than the diameter of the hyphae (Fig. 1C). This was also seen in *H. fasciculare* whereas mitochondria of other fungi were shorter than the hyphal diameter. Mitochondria were in general found lined up after each other along the sides of the membranes. The fluorescence intensity using this dye is proportional to the mitochondrial activity (Liu et al. 1987). Esterase activity or the esterase-cleaved fluorescein was sequestered in vesicles in *H. fasciculare* and in most other fungi tested (Fig. 1D). This is in contrast to conventional FDA staining which leaves the whole cytoplasm fluorescent.

The effect of a total extract of metabolites from *P. fluorescens* DR54 on *R. arrhizus* is shown in Fig. 2. The fungus is stained for detection of hydrophobic membranes or wall structures and storage lipid drops. There is a clear difference between treated and non-treated hyphae. In the control (Fig. 2A), large lipid bodies are present while much less yellow fluorescence is emitted from the treated hyphae (Fig. 2B).

Together with calcofluor white and a pH indicator stain, the four stains reported in Figs. 1 and 2 were used for monitoring the physiological effects of purified viscosinamide from the antagonistic *P. fluorescens* strain DR54 (C. Thrane, T.H. Nielsen, J. Sørensen, and S. Olsson, *unpublished data*). In summary the microscopic observations of relatively slow responses were: Increased swelling and branching of hyphae, changes in membrane/wall hydrophobicity, and occurrence of storage material, and changes in nuclei distribution and size. Medium-fast responses were visualized by staining for structure, localization, and activity of mitochondria and by staining for metabolic (esterase) activity. Initial studies with the pH indicator stain indicated its applicability for detection of a fast response in the fungi (M. Hansen, C. Thrane, J. Sørensen, and S. Olsson, *unpublished data*).

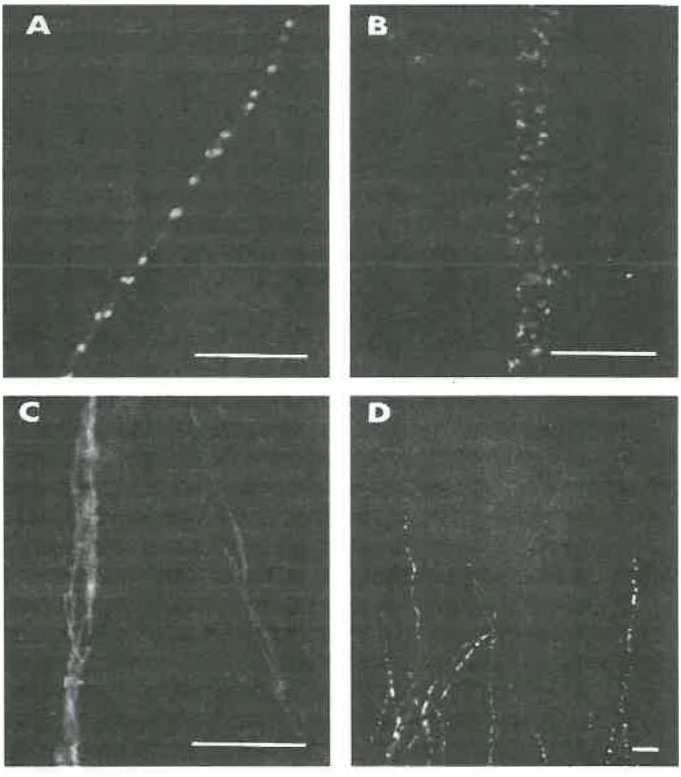

Fig. 1. Staining of nuclei in *Pythium oligandrum* with Syto 13 (A), hyphal hydrophobic structures (membranes and lipid bodies) of *Rhizopus arrhizus* stained with Nile red (B), mitochondria in *Antrodia vaillantii* stained with $DiOC_7(3)$ (C), and, finally, CFDA was used for detection of esterase activity in *Hypholoma fasciculare* (D). Bar = 20 μm.

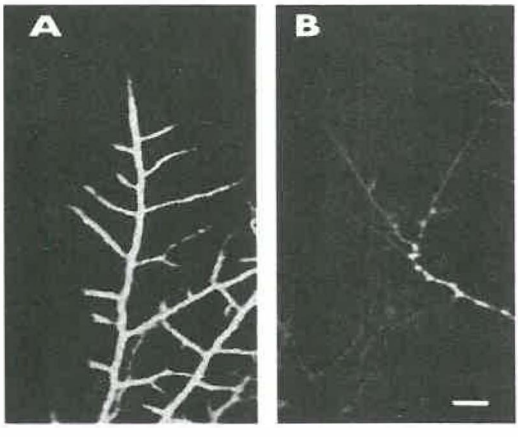

Fig. 2. *Rhizopus arrhizus* challenged with antifungal metabolites from a crude extract of a culture filtrate from *Pseudomonas fluorescens* DR54. By staining of hydrophobic structures with Nile red a large difference in tip membrane hydrophobicity and occurrence of lipid drops is seen between the untreated control (A) and the treated hyphae (B). Bar = 10 μm.

There are only a few mycological studies which have exploited the use of vital fluorescent stains. Yuan and Heath (1991) found that fluorescent membrane probes for studies of hyphal tip physiology in *Saprolegnia ferax* were superior to transmission electron microscopy (TEM) being less prone to artifacts and yielding dynamic information. Habel et al. (1991) studied the stress response of *Neurospora crassa* to elevated temperatures using a fluorochrome that stained mitochondria. Reed et al. (1997) tested the response of different plant pathogenic fungi, including *Pythium* and *Rhizoctonia,* to synthetic bioactive peptides. They used a cell-impermeable stain to study if changes in membrane integrity were induced, and calcofluor white to test if the cell walls remained intact. Stewart and Deacon (1995) studied the possibilities of using fluorescent stains in soil systems. They claimed that, prestaining of fungal inocula and subsequent inoculation in the soil may be useful in studies of fungal ecology. By labelling individual fungi with fluorochromes representing different emission spectra, it may be possible to study competition or interaction between fungi over relatively large periods of time. Our results indicate the possibility of using a single dye for staining of different fungi and still be able to clearly distinguish the fungi in interaction studies.

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Secondary metabolite and endochitinase dependent antagonism against plant pathogenic microfungi in *Pseudomonas fluorescens* isolates from the sugarbeet rhizosphere

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Abstract

Selection and development of biocontrol agents depend on reliable screening procedures and understanding of the mechanisms of disease suppression *in planta*. Fifty-six isolates representing all biovars of *P. fluorescens* (bv. I-VI) were collected from the rhizosphere of field-grown sugar beet plants in order to select candidate strains for biocontrol of pre-emergence damping-off disease. The isolates were tested for *in vitro* antagonism against the plant pathogenic microfungi *Pythium ultimum* and *Rhizoctonia solani* in three different plate test media. Mechanisms of fungal inhibition were elucidated by tracing secondary metabolite production and cell-wall degrading enzyme activity in the same media.

Most biovars expressed a specific mechanism of antagonism, as represented by a unique antibiotic or enzyme production in the media. A lipopeptide antibiotic, viscosin, was produced independently of medium composition by *P. fluorescens* bv. I, whereas the 2,4-diacetylphloroglucinol (DAPG) antibiotic was only observed in glucose-rich medium and only in *P. fluorescens* bv. II/IV. Both pathogens were inhibited by the two antibiotics. An unidentified Fe-regulated compound was only produced in seed extract medium by isolates of *P. fluorescens* bv. III and was only active against *P. ultimum*. Finally, in low-glucose medium, a cell wall-degrading endochitinase activity in both *P. fluorescens* bv. I, III and IV was the apparent mechanism of antagonism against *R. solani*. Strains representing the antibiotic-producing biovars were tested in a pot experiment with sugar beet seedlings. The viscosin-producing DR54 isolate (bv. I) was the most effective in reducing damping-off incidence compared to an untreated control.

Molecular approaches to determine the role of pre-emptive carbon source use in the biocontrol of bacterial speck

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Abstract

The hypothesis is being tested that pre-emptive biocontrol efficacy of a nonpathogenic bacterium is proportional to nutritional similarity between the nonpathogen and the target pathogen. Pre-emptive biocontrol efficacy of forty naturally-occurring nonpathogenic bacteria against bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, was determined in greenhouse assays. When carbon sources presumed to be present in leachates on tomato leaves were used to determine carbon sources utilization profiles of these bacteria, there was a weak positive correlation between nutritional similarity and efficacy. Multiple correlation analyses of the carbon source utilization data and the biocontrol efficacy data suggested that the ability to utilize certain organic acids was highly correlated with biocontrol. When nutritional similarity between the nonpathogens and the pathogens was based upon only these carbon sources, there was a highly significant positive correlation between nutritional similarity and efficacy. The relative abundance of these particular carbon sources on tomato leaves is being analyzed by HPLC with the intention that abundance will be incorporated into estimates of nutritional similarity.

The pre-emptive biocontrol efficacy of Tn *5*-induced catabolic mutants of biocontrol agent *P. syringae* TLP2 is also being determined to provide an independent assessment of the correlation between nutritional similarity and efficacy. The wild-type parent TLP2 utilizes almost all the same carbon sources as the pathogen *P.s.* pv. *tomato* PT12. The catabolic mutants have lost the ability to catabolize one or more carbon sources; hence, while the nutritional similarity between *P. syringae* TLP2 and *P.s.* pv. *tomato* PT12 is approximately 1.0 the nutritional similarity between the catabolic mutants and *P.s.* pv. *tomato* PT12 ranges from about 0.2 to <1.0. There is a significant positive correlation between epiphytic population size of the catabolic mutants and the number of carbon sources utilized. Preliminary data suggest that nutritional similarity between the catabolic mutants of the biocontrol agent *P. syringae* TLP2 and *P.s.* pv. *tomato* PT12 is significantly correlated with pre-emptive biocontrol efficacy. In the future, selected catabolic mutants of *P. syringae* TLP2 [those which are altered in the ability to utilize organic acids or other carbohydrates indicated to be important in the multiple correlation analyses; significantly reduced in colonization capacity in the tomato phyllosphere; and significantly reduced in pre-emptive biocontrol efficacy] will be complemented *in trans* using a genomic library of TLP2 and marker exchange mutants will be constructed to confirm the contribution of the mutated genomic region to the altered phenotype.

Mechanisms of action: fungal agents

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Advances in understanding the antifungal mechanism(s) of *Trichoderma* and new applications for biological control

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Abstract

Significant new results have been obtained in the field of biological control of plant diseases utilizing various fungal agents, in particular *Trichoderma* spp. The antagonistic system of these fungi has been studied in detail and shows a high level of complexity and versatility. These data are enabling elucidation of the different phases of the *Trichoderma*-host interaction, including host recognition and differential activation of genes involved in mycoparasitism. The role and activity of enzymes and antibiotics has been studied extensively, and several synergistic interactions between different components of the *Trichoderma* system have been identified. However, despite an intense research effort, there remains a number of questions concerning the biocontrol mechanism(s). Dissection of the *Trichoderma* system is advancing our understanding, as well as providing genes, promoters, and gene products useful for the improvement of antifungal activity in plants, other biocontrol agents, and *Trichoderma* itself. This review discusses some of the major advances made recently in this field.

Introduction

The increase of food and fiber yield achieved during the past decades is due to the extensive application of pesticides and nitrogen-based (natural or synthetic) fertilizers combined with the development of plant varieties with higher yield production and pest resistance. However, much of this technology is approaching its limit due to constraints of the agriculture system and to the increasing environmental concerns of the public. New tools are being developed which include the genetic manipulation of plants and microbes, and the application of biocontrol agents for disease control. While some bacterial biocontrol agents (i.e., *Agrobacterium* and *Bacillus*) have been successfully exploited at a practical level, biocontrol fungi are only recently moving from the laboratory to the field. The reason may be attributed to the fact that the biocontrol mechanisms of fungal agents are not well understood, as in the case of *Trichoderma* spp., which includes some of the most studied and commercially marketed biocontrol fungal agents. Despite 70 years of research and hundreds of papers describing the efficacy of *Trichoderma* spp. in controlling various plant pathogens, only in the past few years has its antifungal mechanism(s) been investigated in depth. Molecular techniques have been of substantial utility in dissecting the *Trichoderma* system and have revealed an astonishing level of complexity in aspects concerning host recognition, activation of antagonistic genes and mechanism of action of secreted antifungal compounds. This paper briefly reviews some of the recent research on the *Trichoderma*-host interaction, and discusses the potential use of the *Trichoderma* genome to improve plant disease resistance and biocontrol.

The *Trichoderma* system

Trichoderma strains have the ability both to control plant fungal diseases and enhance plant growth (Chet 1987, Whipps 1997). This is the result of a complex interaction between the biocontrol fungus, plant, pathogen(s) and other microorganisms that colonize the soil, rhizosphere, spermosphere and phyllosphere.

The *Trichoderma*-plant interaction and its importance for biocontrol is unclear. Researchers have observed a tight attachment of *Trichoderma* hyphae or spores to plant roots, possibly because of cortex penetration and endophytic growth (Kleifeld and Chet 1992). The specific ability of biocontrol strains to colonize the root and leaf surface (rhizosphere or phyllosphere competence) is still debated and could be a characteristic of particular strains rather than a general case (Green 1997, Harman et al. 1993, Whipps 1997). In addition, it has been noted that *Trichoderma* strains can induce Systemic Acquired Resistance (SAR) in the plant and that SAR may have a role in biocontrol (G. De Meyer and M. Höfte *personal communication*). The mechanism by which several *Trichoderma* strains clearly improve plant growth and crop yields is receiving increasing attention for the potential use of these fungi as biofertilizers. The modes of action involved in plant growth promotion are also unclear and may depend on biocontrol of minor pathogens, production of growth regulators, nitrogen fixation, siderophore activity and/or availability of additional nutrients for the plant (reviewed by Whipps 1997).

Little is known about the interaction between *Trichoderma* and other beneficial microbes such as *Pseudomonas* and mycorrhiza-forming fungi, which may be an important component of the system and modulate biocontrol efficacy. Most of the available data comes from biocontrol tests with combinations of different fungal and bacterial agents, such as *T. harzianum, T. koningii, T. virens* or *Trichoderma* spp. with *P. putida, P. fluorescens* or *Streptomyces griseoviridis*. In most cases, biocontrol level was not affected or enhanced by the use of more than one strain. The formation of arbuscular mychorrhiza was also, with few exceptions, not greatly influenced by the inundative treatment with biocontrol agents (reviewed by Whipps 1997). Such a complex interaction between *Trichoderma* and other beneficial microorganisms needs further study to understand the ecology of these fungi and to develop individual strains or mixtures of biocontrol agents.

The parasitic/antagonistic interaction between *Trichoderma* and pathogenic fungi has received much more attention, since the original reports of Weindling (1934) in the first half of this century. Fungal antagonism involves competition for nutrients and physical niches, antibiosis with production of a variety of antifungal compounds, and direct parasitism (mycoparasitism or hyperparasitism) (Cook and Baker 1983). The importance and the role of these activities in the biocontrol mechanism of *Trichoderma* is still debated. There seems to be major differences among *Trichoderma* biocontrol strains which may preferentially act by antibiosis, mycoparasitism or competition, or by any combination of these activities. One way to schematically describe the antifungal system of *Trichoderma* may be as follows.

Phase 1: Initial interaction and recognition with the host. This phase may be seen as the turning point, changing from the saprophytic to the antagonistic state. The recognition and activation phase may occur before or after physical contact between the two fungi, and may depend on a specific interaction at a molecular level. In addition, nutrient depletion or competition for substrates may also be involved in triggering a parasitic response. Application of new techniques has provided interesting data concerning this phase of the interaction. We have prepared *T. harzianum* mutants expressing the green fluorescent protein (GFP) from

Aequorea victoria under the promoter of endochitinase-encoding gene *ech42* (Lorito et al. 1996a, Zeilinger et al. *unpublished data*) and the promoter of the N-acetyl-β-glucosaminidase-encoding gene *nag1* (Peterbauer et al. 1996), both cloned from *T. harzianum* and inducible by the mycoparasitic interaction. These promoter sequences allowed the mutants to become fluorescent during the interaction with the host, following the expression of the native enzymes during antagonism. Interestingly, the endochitinase promoter was activated much before contact occurred between *Trichoderma* and its host *Rhizoctonia* and this could be prevented by separating the two fungi with a 12,000 kDa cutoff dialysis membrane (Zeilinger et al. *unpublished data*). These results indicate that host recognition and activation of genes involved in mycoparasitism, antibiosis or antagonism occur at a distance, and that diffusible macromolecules are involved in the recognition and activation of parasitism with production of antibiotic substances. Receptors and elicitors, which are most likely involved in this phase, have not been identified yet. However, *Trichoderma* mutants containing reporter genes (GFP) under the control of promoters turned on during mycoparasitism should be of substantial utility for elucidating this aspect (Lorito et al. 1996a).

Trichoderma may grow chemotactically towards the host, possibly attracted by metabolites or molecules released from the host cell wall by the action of *Trichoderma* cell wall degrading enzymes (CWDEs) that have been secreted constitutively at low level. Nutrient competition and starvation may also cause an increase in CWDEs secretion, thus enhancing the ability of *Trichoderma* to detect a suitable host and initiate parasitic behavior. In addition, Chet and co-workers clearly indicated that an important part of the recognition process can occur during the physical interaction of a *T. harzianum* strain with its host (Inbar and Chet 1992, 1995). By using a "biomimic" system, it was demonstrated that a specific lectin-sugar interaction triggers mycoparasitic events, such as coiling and formation of appressoria-like structures. Moreover, these authors discovered that recognition of different hosts activated different sets of chitinolytic enzymes and modulated the mycoparasitic response (Haran et al. 1996).

Phase 2 - Physical and molecular attack against the host. During this phase, *Trichoderma* secretes a complex mixture of antifungal compounds, enzymes and/or antibiotics, then physically coils around the host mycelium and binds to it firmly, forms appressoria-like structures and perforates the host wall (Chet 1997). These are very complex phenomena, which involve a cascade of different events depending on the *Trichoderma* strain and on the host species (Haran et al. 1996). Some strains may have the ability to attack the host biochemically before contact by using antibiotics (Belanger et al. 1995), enzymes or combinations of both, and then saprophytically colonize the dead host cells. Others may require physical contact for a full activation of the mycoparasitic cascade, or rely more on the antifungal CWDEs and less on the antibiotics to attack the host (Belanger et al. 1995, Benhamou and Chet 1993, M. Lorito and G. Harman *unpublished data*).

A great variety of genes and compounds are involved at this phase. *Trichoderma* is able to secret endochitinase, chitobiosidase, N-acetyl-β-glucosaminidase, N-acetyl-β-galactosaminidase, β-1,3-glucanase, β-1,6-glucanase, protease, DNAse, •-amylase, cellulase, lipase, mannanase, xylanase, urease, RNAase, pectinase, pectin lyase, laccase, peroxidase and mutanase, etc. (reviewed by Lorito 1998). The activation of a different array of genes is required to produce the morphological changes in the *Trichoderma* mycelia for the coiling, pressuring, and penetration of the host. Finally, a number of other genes are involved in the production of a variety of antibiotics, such as peptaibols, gliotoxin, pyrones and others (Howell 1987, Scarselletti and Faull 1994, Sivasithamparam and Ghisalberti 1998, Solfrizzo et al. 1994).

Extensive work has been done to characterize chitinolytic, glucanolytic and proteolytic enzymes, and their genes, purified from biocontrol strains of *Trichoderma* (reviewed by Chet et al. 1998, Benitez et al. 1998, Lorito 1998). Some of these enzymes have a strong antifungal activity, especially when assayed in combinations (Lorito et al. 1993a-c, 1994a-c, 1996b-d). CWDEs are produced concurrently with powerful antibiotics during mycoparasitism and act synergistically with peptaibols and gliotoxin (Di Pietro et al. 1993, Lorito et al. 1994b, 1996b, 1996d, Schirmböck et al. 1994). The synergistic interaction between enzymes and antibiotics uses the host cell wall as a primary target. The enzymes act on the chitin/cellulose-glucan complexes while the antibiotics affect the integrity of the host membranes as well as the associated chitin and glucan synthase activities (Lorito et al. 1996d). However, the role of the various antifungal compounds during the parasitic phase and biocontrol is still unclear and many questions remain unsolved. While the function of an antibiotic in biocontrol has been investigated with gliotoxin negative mutants of *T. virens* (Howell 1987), studies are ongoing to assess the importance of CWDEs in the *Trichoderma* mechanism. *T. harzianum* disrupted mutants with CWDE activities blocked or reduced, as well as mutants with enhanced CWDE activities, have been recently obtained in different laboratories (Flores et al. 1996, M. Lorito and S. Woo *unpublished data,* Margolles-Clark et al. 1996a). The comparative assays of these mutants and the wild type strain for in vivo biocontrol of plant pathogens should determine the role of each enzyme and provide useful indications for strain improvement programs.

Also intriguing is the regulation of the expression of the various genes encoding antifungal compounds during mycoparasitic attack. Most of the work has been done with the recently cloned genes for chitinolytic and glucanolytic enzymes, which are expressed at different times and in different combinations depending on the strains involved (Garcia et al. 1994, Haran et al. 1996, Hayes et al. 1994, Lorito et al. 1993a, Peterbauer et al. 1996). The analysis of promoter regions and the use of reporters should be very useful to understand the sequence of gene activation events and to study gene regulation. For instance, we have successfully studied the interaction of the promoter region of the *ech42* (endochitinase-encoding) gene with various DNA-binding and regulatory factors during mycoparasitism of *Botrytis cinerea* by *T. harzianum* (Lorito et al. 1996a). We also used this promoter and the promoter region of *nag1* (Peterbauer et al. 1996) to demonstrate the differential activation of the two CWDE genes, which occurred before (*ech42*) and after (*nag1*) the contact between *T. harzianum* and *R. solani* (Zeilinger et al. *unpublished data*). The cloning and the characterization of more "biocontrol" genes and related promoters should allow the in vivo study of the genetic events in the *Trichoderma*-host and *Trichoderma*-plant interaction.

An open question is how does the mycoparasite protect itself from the powerful synergistic mixtures of antifungal compounds secreted during antagonism and from antibiotics and enzymes produced by the host? Probably *Trichoderma* uses cell-wall-bound inhibitors for a few key enzyme activities, thus reducing the effect of the entire synergistic mixtures at the concentrations used during the parasitic attack. Lora et al. (1994) found a cell-wall-bound chitinase inhibitor induced simultaneously with CWDEs and putatively involved in a self-protection mechanism, which may explain the high level of resistance of *T. harzianum* mycelia to its own purified endochitinases (Lorito et al. 1993b). On the other hand, antibiotic resistance may be accomplished in *Trichoderma* by membrane-bound proteins able to detoxify the cell by pumping out toxic compounds (M. Lorito and G. Del Sorbo *unpublished data*). This is in accordance with the observed high level of resistance of biocontrol *Trichoderma* strains to several chemical fungicides (G. Harman *personal communication*). Clearly, self-protection from endogenously produced antifungal compounds

may become a key issue if strain improvement for biocontrol is attempted by enhancing or deregulating the production of CWDE and/or antibiotics.

Phase 3 - Host colonization and disruption. In the last phase, mycoparasitic *Trichoderma* penetrates the host mycelium and actively multiplies inside the cell, leading to a complete breakdown of the parasitized fungus. Chitinolytic, glucanolytic and cellulolytic enzymes sustain this action of the mycoparasite in two ways: i) CWDEs locally soften the cell wall allowing *Trichoderma* hyphae to penetrate with little mechanical pressure; ii) CWDEs extensively alter the host cell walls which permits the concurrently produced antibiotics to penetrate in substantial amounts and exert their disruptive action on the plasmalemma and other cell membranes. This synergistic mechanism of interaction between CWDEs and antibiotics has been described recently by Lorito et al. (1996c, 1996d), and may also concern other cases (i.e., plant-fungus interaction) in which CWDEs and antifungal toxins are simultaneously used (Lorito et al. 1996b). During this phase, which ends with the disruption of the target fungus, full expression of genes encoding for specific CWDEs such as exo-β-1,3 glucanases, β-1,6 glucanases, lipases, proteinases and permeases is probably required both to release and to assimilate nutrients from the host tissues (M. Lorito and B. Donzelli *unpublished data*, Vasseur et al. 1995). It is interesting to consider that synergistic combinations of *Trichoderma* CWDEs are able to lyse completely not only mycelia, but also sclerotia and other strong host tissues (Benhamou and Chet 1996) making these enzymes and their genes very attractive for direct application against fungal pathogens (Lorito et al. 1996e).

New applications in biocontrol

Trichoderma strains are marketed today as biofungicides for disease control of vegetables, flowers and trees under certain conditions, and also in combination with chemical pesticides (Harman and Hayes 1996). A realistic application of these biocontrol strains is to complement pesticide use in bio-chemical formulations and provide protection where chemicals are ineffective or not permitted.

Some new and exciting applications concerning *Trichoderma* are being developed. Synergistic combinations of CWDEs are being tested for the preparation of new fungicidal formulations containing low levels of chemical pesticides and enzymes. Therefore, production levels of powerful, stable and low-cost antifungal enzymes are being brought up to a commercial scale by genetic engineering (Margolles-Clark et al. 1996b). In addition, the cloning of *Trichoderma* genes encoding for CWDEs allows the use of specific components of the molecular arsenal of these mycoparasites as an alternative to the application of the whole biocontrol organism. Recently, Lorito and co-workers (1996f, 1998, and *unpublished data*), and other researchers have transferred *Trichoderma* genes coding for CWDEs into different crop plants, including tobacco, potato, tomato, brassica, apple and petunia. Some transgenic lines have shown increased resistance to foliar and soilborne pathogens even with the introduction of a single *Trichoderma* gene (Lorito et al. 1998). Interestingly, preliminary results indicate that the transgenic enzyme does not act exclusively as an inhibitory agent, but activates the plant defense response by releasing elicitors from the wall of the invading fungus (M. Lorito, S. Woo, and F. Scala *unpublished data*). In addition, the transformation of an *Enterobacter cloacae* strain with the *T. harzianum* endochitinase-encoding gene has been used to improve biocontrol ability (G. Harman *personal communication*, Lorito et al. 1993c).

CWDEs genes are also being applied to improve biocontrol ability in *Trichoderma* by using strong promoters and increased gene copy numbers (Flores et al. 1996, Margolles-Clark

et al. 1996a). Another powerful tool is provided by the cloning and the characterization of "mycoparasitic" promoters of *Trichoderma* (Lorito et al. 1996a, Peterbauer et al. 1996). Recently cloned CWDEs promoter regions were able to strongly activate heterologous genes in *T. harzianum* mutants only in the presence of the host (Zeilinger et al. *unpublished data*). Therefore, these inducible promoters could be used to express any useful genes in *Trichoderma* during mycoparasitism and to improve biocontrol ability.

Together these results clearly indicate that the *Trichoderma* "antifungal" system may be a good source of genes and promoters, as well as antibiotics and enzymes, for controlling pathogenic fungi.

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Preliminary study of exo-β-1,3-glucanase encoding genes in relation to the protective activity of *Pichia anomala* (strain K) against *Botrytis cinerea* on postharvest apples

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Abstract

In a previous study, the yeast *Pichia anomala* strain K was found to provide a high and reliable level of protection against *Botrytis cinerea* on wounded apples. An exo-β-1,3-glucanase (paexg2), active in reducing *B. cinerea* germinative tube growth, was purified to homogeneity from a culture filtrate of strain K. In order to find genes coding for exo-β-1,3-glucanase in strain K, a PCR approach using degenerated primers was used. Two PCR fragments (*PAEXG*1a and *PAEXG*2a) were found to share a significant similarity at the deduced amino acid level with exo-β-1,3-glucanases from other fungi. *PAEXG*1a and *PAEXG*2a have 90% identity in their homologous region at the amino acid level and 80% identity at the nucleotide level. Haploid populations have been produced from the diploid strain K by ascus dissection. *PAEXG*1a and *PAEXG*2a served as probes in Southern blots on genomic DNA extracted from strain K and its haploid descendants. Segregation of *PAEXG*1a and *PAEXG*2a was discussed in relation to the in vivo protective effect and the in vitro exo-β-1,3-glucanase activity of the yeasts.

Introduction

Botrytis cinerea Pers.:Fr. is one of the most devastating pathogens of postharvest apples. Its control is based primarily on chemical fungicides. Biological control of postharvest diseases appeared to be a realistic approach because of the defined and stable environmental conditions in storage rooms and the high value of some harvested commodities. Numerous microbial strains exhibiting an antagonistic activity against *B. cinerea* have been reported in the literature (Wilson and Wisniewski 1989). Jijakli (1996) isolated the yeast *Pichia anomala* (Hansen) Kurtzman strain K from the surface of an apple and demonstrated that it exhibits a high and reliable biocontrol activity against infection of *B. cinerea* on wounded Golden Delicious apples at 5 °C and 25 °C.

Knowledge of the mechanisms of antagonism operative in biocontrol may be helpful in enhancing the antagonistic efficiency. However, reports postulating mechanisms of antagonism used by yeasts in gray mold control on apple have been few. These include preemptive exclusion by yeasts of fungal infection sites, competition for nutrients and mycoparasitism (Mercier and Wilson 1994, Roberts 1990, Janisiewicz et al. 1994, Wisniewski et al. 1991). Elucidation of the mechanisms of action are often hampered by the complex interactions between host-pathogen-antagonist. Moreover, the mechanisms studied in vitro do not necessarily reflect in situ activity.

An exo-β-1,3-glucanase activity has been measured in culture filtrates of strain K grown in the presence of a *B. cinerea* cell wall preparation. An exo-β-1,3-glucanase (paexg2)

purified to homogeneity from strain K culture filtrate showed in vitro inhibitory effects on germinative tube growth of *B. cinerea*. An exo-β-1,3-glucanase activity was detected on strain K-treated apples and might be related to paexg2. Moreover, the addition of *B. cinerea* cell wall to suspension of *P. anomala* stimulated both in situ exo-β-1,3-glucanase activity and protective activity against the pathogen (Jijakli 1996). Overall results suggested that the exo-β-1,3-glucanase activity might be involved in the protective activity of strain K. However, experimental evidence of such a role is still missing and would require genetic studies of the yeast.

In this respect, this work aims to study exo-β-1,3-glucanase encoding genes in the genome of strain K and its haploid descendance in relation to the protective activity against *B. cinerea* on wounded apples and the in vitro exo-β-1,3-glucanase activity production of those yeasts.

Materials and Methods

Strains and growth conditions. *Botrytis cinerea* Pers.:Fr. strain V was isolated from a rotting strawberry and stored on oatmeal agar (oatmeal 4.5%, boiled for 30 min, 2% agar) at 4 °C in the dark. The pathogen was cultivated on oatmeal agar at 21 °C for 8 to 12 days. Conidial suspension was obtained as described by Jijakli (1996) and adjusted to 10^6 spores per ml. And *B. cinerea* cell wall preparation was prepared with a method derived from the method described by Jijakli (1996).

Pichia anomala (Hansen) Kurtzman strain K was isolated from an apple in its diploid form. It was induced to sporulate on 1% potassium acetate, pH 6.5, 1.5% agar at 20 °C for 8 days. Ascospores were isolated by micromanipulation on YEPD-agar after treatment with lyticase (from *Arthrobacter luteus*, Sigma Chemical Co., St Louis, MO). Ten haploid segregants from strain K (strain Kh 1 to 10) have been used in this study. All yeasts were stored on YEPD-agar at 5 °C. Cells suspensions were prepared by subculturing the yeasts three times at intervals of 24 h on YEPD-agar at 25 °C. Cells from the third culture were suspended in sterile isotonic water (0.85% NaCl). Yeast concentrations were determined spectrophotometrically at 595 nm following a standard curve and adjusted to the desired concentration.

Purification and sequencing of an exo-β-1,3-glucanase from strain K. An exo-β-1,3-glucanase (paexg2) was purified from a culture filtrate of strain K grown in yeast nitrogen broth (YNB, Difco) supplemented with 0.2% *B. cinerea* cell wall preparation as described by Jijakli (1996). The N-terminus was sequenced by Wattiez R (UMH, Mons, Belgium).

Nucleic acid preparation. Yeast genomic DNA was prepared by a glass beads disruption method described by Ausubel et al. (1991). PCR reactions were performed in 50 µl volume of 1 x PCR buffer (Boehringer Mannheim GmbH, Germany) containing 100 ng of yeast genomic DNA, 1 µM of forward and reverse primers (Pharmacia Inc., Piscataway, NJ), 200 µM of each dNTP, 1 unit of Taq DNA polymerase (Boeringer). Denaturation of the template was carried out at 94 °C for 5 min. The following thermal cycling scheme was used for 40 reaction cycles (PTC-200, MT Research, Watertown, MA): 94 °C for 1 min, 45 °C (cycles 1-5) or 50 °C (cycles 6-40) for 30 s, 72 °C for 30 s. A final 10-min elongation step was performed at the end of the cycles. PCR amplification products were size fractionated by 1% agarose gel electrophoresis in TAE buffer (Maniatis et al. 1982). Bands of expected lengths were excised and eluted with the QIAEX gel extraction kit (Qiagen Inc., Chatsworth, CA) and cloned into the pCR™II plasmid with the TA cloning kit (Invitrogen, San Diego, CA) according to

manufacturer's instructions. The nucleotide sequence of the cloned PCR fragments were obtained by the enzymatic dideoxy chain termination method with the T7 Sequencing kit (Pharmacia) according to provided instructions.

Two PCR degenerate primers [Pichia 1] and [Pichia 3as] were designed from conserved amino acid regions found in exo-β-1,3-glucanases of several yeasts (EXG from *Hansenula polymorpha* (Genbank accession code: Z46868), *Kluyveromyces lactis* (Z46869), *Schwanniomyces occidentalis* (Z46871), *Yarrowia lypolitica* (Z46872), EXG1 (M34341) and SPR1 (S52932) from *Saccharomyces cerevisiae*, XOG from *Candida albicans* (X56556) and one filamentous fungus (EXG1 of *Agaricus bisporus* (X92961)). The forward primer [Pichia 1] was 5'-GCATCCCNATHGGNTAYTGG-3' (N = A+C+G+T, H = A+T+C, Y = C+T) and coded for the amino acid sequence IPIGYW. The reverse primer [Pichia 3as] was 5'-GARTTRTCRAANCCRTTYTG-3' (R = A+G) and was complementary to the sequence coding for QNGFDN. The forward degenerate primer [Pichia 7] was 5'-GATAARTTTCGGGGNGTN-3' and corresponded to the amino acid sequence DKFRGV from the N terminal sequence QPWRQQNDKFRGVNLG of paexg2.

Southern blots were performed as follows: approximately 1µg of DNA was digested by restriction endonucleases in 2X One Phor All (OPA) buffer (all enzymes and 10X OPA buffer were purchased from Pharmacia Biotechnologies). Digested DNA was size fractionated by electrophoresis in 0.5% agarose gel and transferred to positively charged nylon membranes (Boeringer) by vacuum blotting. Blots were hybridized to DNA probes P^{32}-labelled with a multiprime DNA labelling kit (Amersham International plc, Bucks, UK). Prehybridization was performed in 10x Denhardt's, 3 x SSC, 0.1% SDS, 0.01% herring sperm DNA, at 65 °C for 2 h. Hybridization was performed overnight at 65 °C in the same solution containing the probe. Membranes were submitted to 2 washes (15 min and 30 min) in 2 x SSC, 0.1% SDS and 2 washes (15 min and 30 min) in 0.2 x SSC, 0.1% SDS.

Enzyme assays and protein measurements. 50 ml YNB supplemented with 0.2% *B. cinerea* cell wall preparation as sole carbon source were inoculated with 2.10^7 CFU of the tested yeast. Flasks were shaken at 25 °C for 5 days at 100 rpm. Culture filtrates were collected after centrifugation at 10,000 rpm for 10 min at 4 °C and extensively dialyzed against 0.05 M potassium acetate buffer (pH 5.5) at 4 °C. For enzyme activity measurements, 250 µl of 0.05 M potassium acetate (pH 5.5) containing 1% laminarin (from *Laminaria digitata*, Sigma) were added to 250 µl culture filtrate. This reaction mixture was incubated with gentle agitation at 50 °C for 16 h. The exo-β-1,3-glucanase (EC 3.2.1.-58) activity was assayed from 100 µl reaction mixture by following the release of free glucose from laminarin with a commercial glucose oxydase kit (Sigma) using glucose as a standard according to manufacturer's instructions. One unit (U) of exo-β-1,3-glucanase activity was defined as the amount of enzyme releasing 1 µg of glucose equivalent per minute, per millilitre of enzyme solution. Specific activity was expressed in units per mg of protein (SU). The experiment was repeated once independently (two culture filtrates per yeast). Results of the two experiments were submitted to variance analysis and means were separated by the Fisher's least significant difference at $P \leq 0.01$ with the SYSTAT software (SYSTAT Inc., Evaston, IL, USA).

Biological protection assays. Apple fruits (cv. Golden Delicious) were brought in a commercial store and maintained at 4 °C until used. Fruits were surface-disinfected with 10% sodium chloride for 2 min, rinsed with sterile distilled water and wounded with a cork borer (two wounds of 6 mm diameter and 3 mm deep at the equator of each fruit). The wounds were treated with 50 µl of a suspension of each yeast (in isotonic water or in an aqueous suspension of 0.2% cell wall preparation) or with 50 µl isotonic water as a control. The fruits were left in

closed plastic boxes with a wetted filter paper at 21 °C in darkness. After 24 h, the wounds were inoculated with 50 µl of a *B. cinerea* conidial suspension. Fruits were incubated in the same conditions for 7 days before measuring diameters of decay lesions. Five apples were used per treatment. Each experiment was conducted twice independently. Results were subjected to analysis of variance and means were separated by the Fisher's least significant difference at $P \leq 0.01$ (first assay) or the Dunnet's test at $P \leq 0.05$ (second assay) with the SYSTAT software.

Results and Discussion

Isolation of *PAEXG*1 and *PAEXG*2 fragments. Degenerate primers [Pichia 1] (sens) and [Pichia 3as] (antisens) based on conserved regions between exo-β-1,3-glucanases from different fungi gave rise to the amplification of a 163 bp DNA fragment (*PAEXG*1a) from strain K genomic DNA, the expected length of the PCR product being about 200 pb. A 390 bp DNA fragment (*PAEXG*2a) was also amplified from strain K genomic DNA with the primers [Pichia 7] derived from the N terminal sequence of the purified paexg2 and [Pichia 3as], the expected length of that PCR product being about 400 bp. The PCR products *PAEXG*1a and *PAEXG*2a were cloned and sequenced (Fig. 1). They both showed a significant similarity, at the deduced amino acid sequence level, with exo-β-1,3-glucanases of other fungi. *PAEXG*1a shared a 76% similarity with the protein coded by XOG of *Candida albicans* and *PAEXG*2a shared 66% with the protein coded by EXG1 of *Saccharomyces cerevisiae*. *PAEXG*1a and *PAEXG*2a shared a 90% identity at the amino acid level and a 80% identity at the nucleotide level in their homologous region. These results suggest that at least 2 exo-β-1,3-glucanase encoding genes (*PAEXG*1 and *PAEXG*2) are present in strain K genome. *PAEXG*2a correspond probably to the gene coding for the purified paexg2. That result corroborates the observation, with native gel detection, of two bands of exo-β-1,3-glucanase activity of different intensity in the culture filtrate of strain K grown with cell walls (Jijakli 1996). The highest activity being produced by paexg2, *PAEXG*1 could then code for an exo-β-1,3-glucanase partly or entirely responsible for the less active band. *PAEXG*1a and *PAEXG*2a were used as probes in Southern blot hybridizations.

PAEXG1a: 1-atccccatcggttactgggcattttatctattagaagatgatccatacgttcaaggtcaagaaccttatttggataaggctttggaa
tgggccaaacagaatgatttgaaagtttggattgacttgcatggtccaggctctcaaaacggatttgataactcaa-163

PAEXG2a: 1-gatatgtttcggggcgttaacttaggtgggtggtttgttttagaaccttttatcactccttctttatttgaagcttttgaaaatcaaggc
caagatgttcctgttgatgaataccattacaccaaagcccttggtaaggatttagctaaagagagattggtcaacattggagttcatggattgttgaag
ctgatttccaaagcattgcaggtgctggtttaaacttttgttcgtattccaatcggttattgggcattccaattgttggataatgatccatacgttcaaggtca
agaatcatatttggaccaagctttagaatgggctaagaaatatgatattaaagtttggattgatttacatggtgctctcagttctcaaaacggcttt-390

PAEXG1a amino acid sequence: LQNSACIPIGYWAFYLLEDDPYVQGQEPYLDKALEWAKQ
NDLKVWIDLHGPGSQNGFDNS

PAEXG2a amino acid sequence: DMFRGVNLGGWFVLEPFITPSLFEAFENQGQDVPVDEYHYT
KALGKDLAKERLDQHWSSWIVEADFQSIAGAGLNFVRIPIGYWAFQLLDNDPYVQGQESYLD
QALEWAKKYDIKVWIDLHGALSSQNGF

Fig. 1. Nucleotide sequences and derived amino acid sequences of the PCR products *PAEXG1*a and *PAEXG2*a.

Analysis of genomic DNA of strain K and its segregants. Genomic DNA extracted from the diploid strain K was cut with 10 restriction enzymes and subjected to Southern blots hybridizations with *PAEXG*1a and *PAEXG*2a as probes. Both probes hybridized to one or two bands depending on the restriction enzyme used (results not showed). Restriction enzymes giving two hybridization bands were chosen (EcoRI, EcoRV for *PAEXG*1a; EcoRV, BamHI for *PAEXG*2a) to treat DNA from strain K and its ten haploid segregants. For the ten segregants, *PAEXG*1a or *PAEXG*2a hybridized to a single band corresponding either to one or the other band detected on strain K genomic DNA blots. Blots with EcoR V digestions are showed in Fig. 2. Repartition of bands obtained on blots with EcoRI and BamHI digestions, probed with *PAEXG*1a or *PAEXG*2a respectively, was the same as on EcoR V blots. So, *PAEXG*1 and *PAEXG*2 turned out to be single copy genes and the two bands observed on strain K genomic blots were due to the diploid status of this yeast. Assuming that the two fragments hybridizing with each probe were representative of two alleles of each *PAEXG*1 or *PAEXG*2 gene (alleles *PAEXG*1-1 and *PAEXG*1-2, alleles *PAEXG*2-1 and *PAEXG*2-2), we may identify four genetic types among the 10 segregants (Table 1). This shows that *PAEXG*1 and *PAEXG*2 segregate independently.

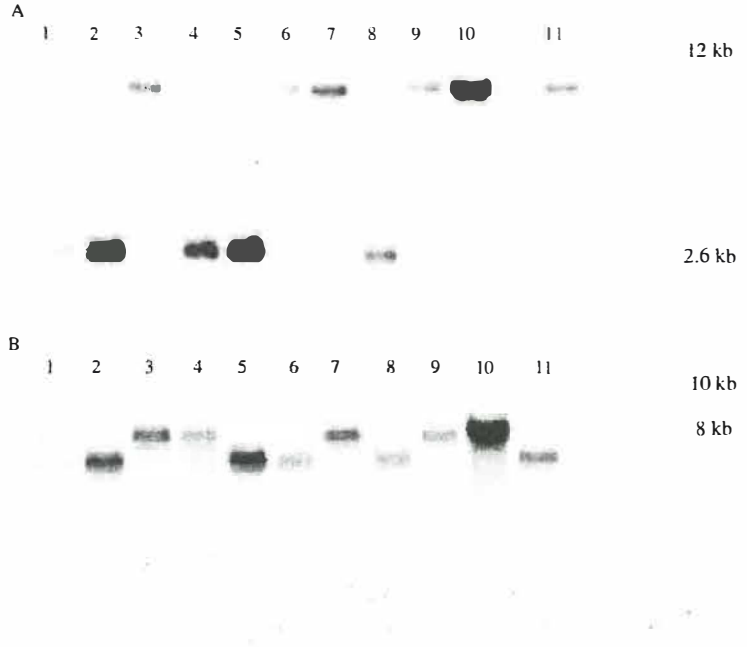

Fig. 2. Blots of total genomic DNA from strain K (lane 1) and its ten haploid segregants strain Kh 1 to 10 (lanes 2 to 11) cut with EcoRV. The blots were probed with P32-labelled PCR products *PAEXG*1a (blot A) and *PAEXG*2a (blot B).

Table 1. Segregation of alleles of the two genes *PAEXG*1 and *PAEXG*2 in the haploid segregants derived from strain K.

Yeast Strain	Alleles			
	*PAEXG*1-1	*PAEXG*1-2	*PAEXG*2-1	*PAEXG*2-2
K	•	•	•	•
Kh 1	•		•	
Kh 2		•		•
Kh 3	•			•
Kh 4	•		•	
Kh 5		•	•	
Kh 6		•		•
Kh 7	•		•	
Kh 8		•		•
Kh 9		•		•
Kh 10		•	•	

Genetic type	Yeast Strain
*PAEXG*1-1 *PAEXG*2-1	Kh 1, Kh 4, Kh 7
*PAEXG*1-1 *PAEXG*2-2	Kh 3
*PAEXG*1-2 *PAEXG*2-1	Kh 5, Kh 10
*PAEXG*1-2 *PAEXG*2-2	Kh 2, Kh 6, Kh 9

Exo-β-1,3-glucanase activity measurements. Strain K and its segregants were grown in presence of *B. cinerea* cell walls as sole carbon source. The in vitro exo-β-1,3-glucanase activity was measured in the culture filtrates. Out of the ten haploid yeasts tested, strain Kh 1 and strain Kh 7 showed a level of exo-β-1,3-glucanase specific activity significantly higher ($P \leq 0.01$) than strain K activity, the others producing the same specific activity level as strain K (Fig. 3). No relation could be found between established genetic types and the different levels of enzymatic activity produced in vitro.

Biological protection assays. The antagonistic activity against *B. cinerea* of strain K and its segregants was tested in vivo on wounded apples. A first experiment was conducted with yeasts inoculated at 10^6 CFU/ml in isotonic water. All yeasts reduced lesion diameter (protection levels between 56 and 77%) (Fig. 4). strain K protection level was consistant with the levels observed in the same conditions by Jijakli (1996). Strain Kh 2, strain Kh 3 and strain Kh 9 protected significantly less ($P \leq 0.01$) than strain K, strain Kh 1, strain Kh 6 and strain Kh 7, the other haploids showing an intermediate protective effect. A second experiment was conducted with each yeast inoculated at 10^5 CFU/ml in isotonic water or in an aqueous suspension of 0.2% cell wall preparation. Strain K, strain Kh 1, strain Kh 5 and strain Kh 6 showed an increased protective effect ($P \leq 0.05$) when inoculated with *B. cinerea* cell walls (Fig. 5). Concerning the other yeasts, the addition of cell walls add no significant effect on the antagonism. For these two experiments, no relation could be found between genetic types and either the different levels of antagonism or the different effects of the addition of *B. cinerea* cell walls on the antagonism.

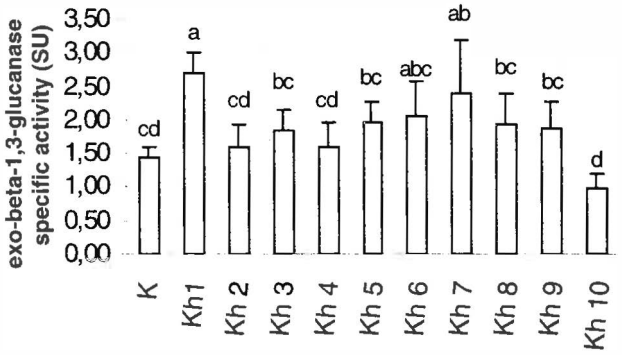

Fig. 3. Mean exo-β-1,3-glucanase specific activities produced by strain K and its segregants (strains Kh 1 to 10) when grown with *B. cinerea* cell walls as sole carbon source. Data from the two repetitions of the experiment were submitted to variance analysis and pooled. Means with the same letter are not significantly different from each other (Fisher's LSD test, $P \leq 0.01$).

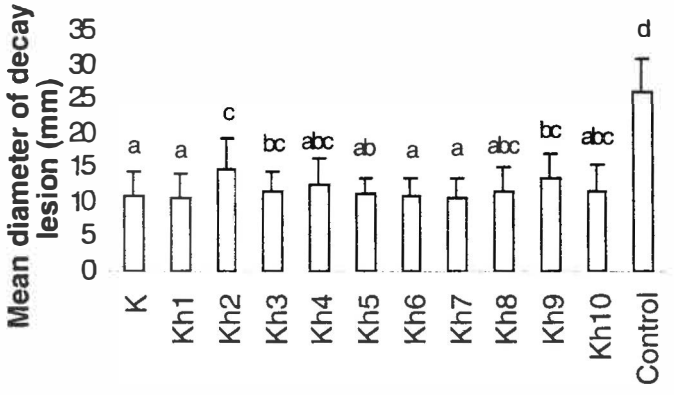

Fig. 4. Mean diameters of decay lesions when apple wounds were inoculated with 50 µl of yeast suspension (strains K and Kh 1 to 10) at 10^6 cfu/ml in isotonic water and 50 µl of *B. cinerea* spore suspension at 10^6 spores/ml at 24 h interval. Data from the two repetitions of the experiment were submitted to variance analysis and pooled. Means with the same letter are not significantly different from each other (Fisher's LSD test, $P \leq 0.01$).

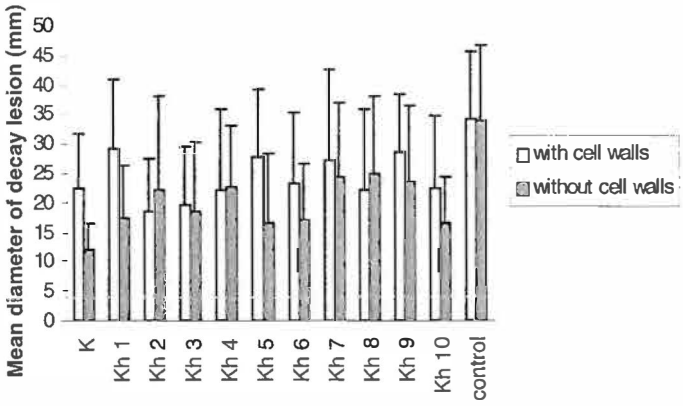

Fig. 5. Mean diameters of decay lesions when apple wounds were inoculated with 50 µl of yeast (strains K and Kh 1 to 10) at 10^5 cfu/ml in aqueous suspension of 0.2% cell wall preparation and 50 µl of *B. cinerea* spore suspension at 10^6 spores/ml at 24 h interval. Data from the two repetitions of the experiment were submitted to variance analysis and pooled.

Conclusion

None of the studied segregants derived from strain K was defective in in vitro exo-β-1,3-glucanase activity production or in protective activity against gray mold on wounded apples. The in vitro enzymatic activity of the segregants was not related to their protective activity. Moreover, segregation of the strain K genes *PAEXG1* and *PAEXG2* influence neither in vitro exo-β-1,3-glucanase activity nor in vivo protective activity of the yeasts postulating that the diploid strain K may be homozygous at those loci. Hence the variations observed between haploid segregants for the studied characteristics point out that other factors (genes or regulating elements) than the genes *PAEXG*1 and *PAEXG*2 are active in the in vivo exo-β-1,3-glucanase production and the protective effect.

Accordingly, the possible implication of the exo-β-1,3-glucanase activity in the protective effect remains to be elucidated. As a first step, the in situ transcription and translation of *PAEXG*1 and *PAEXG*2 will be investigated on apples at the site of *B. cinerea-P. anomala* interaction. Since some haploid segregants derived from strain K produce as much exo-β-1,3-glucanase activity as strain K (or more) and protect apples against *B. cinerea* with the same efficiency as strain K (in the experimental conditions), the implication of *PAEXG*1 and *PAEXG*2 in the antagonism will be further studied through their disruption by integrative transformation in the genome of the haploid material. To reach that purpose, we are currently attempting to isolate *PAEXG*1 and *PAEXG*2 from a strain K genomic library in the phagic vector EMBL3 to sequence them.

Acknowledgments

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Are extracellular enzymes involved in the mycoparasitism of *Pythium mycoparasiticum* and are zoospores of *P. oligandrum* attracted to host colonized and non-colonized roots?

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Abstract

Pythium mycoparasiticum and *P. oligandrum* are aggressive mycoparasites with many similarities. A study of the possible involvement of hydrolytic enzymes in the parasitism of host hyphae and conidia by *P. mycoparasiticum* is in progress. NAGase, endo-chitinase, protease, β-glucosidase and cellobiohydrolase activities were detected in the growth media following co-inoculation of *P. mycoparasiticum* and living hyphae and cell walls of *Fusarium culmorum* and *P. ultimum* and conidia of *F. culmorum*. Endo-cellulase activity was detected when *P. mycoparasiticum* was inoculated to living *P. ultimum* and β-glucanase was detected when *P. mycoparasiticum* was inoculated to living host hyphae or conidia. The results indicated that enzymes produced by both the host and mycoparasite may be involved in the penetration process of host hyphae and conidia by *P. mycoparasiticum*. In contrast to *P. mycoparasiticum, P. oligandrum* produces zoospores and the zoospores of *P. oligandrum* were neither attracted to host colonized nor non-colonized roots. Furthermore they were not attracted to roots of commercially grown pot plants.

Introduction

Pythium mycoparasiticum and *P. oligandrum* are aggressive mycoparasites with many similarities in physiology, morphology, and in the mycoparasitic process (Deacon et al. 1991, Jones and Deacon 1995, Laing and Deacon 1991). *Pythium oligandrum* is distributed all over the world while *P. mycoparasiticum* so far has only been detected in Great Britain. While *P. oligandrum* has received more attention than *P. mycoparasiticum*, microscopical studies showed that *P. mycoparasiticum* is as effective a mycoparasite as *P. oligandrum* (Laing and Deacon 1991). However, only *P. oligandrum* produces zoospores. This is an advantage in mycoparasitism and probably also in biocontrol as zoospores are able to orient towards host hyphae during encystment and to establish on host cultures, however the zoospores are not attracted to host cultures (Madsen et al. 1995). The ability of *P. mycoparasiticum* to colonize roots has not to our knowledge been studied. *Pythium oligandrum* has a relatively good colonization and oospore production on roots colonized by host fungi or diseased roots, but its colonization of young non-colonized roots is poor (Madsen 1996). In spite of this, *P. oligandrum* has in several investigations successfully controlled diseases caused by several soilborne pathogens (McQuilken et al. 1992). *Pythium mycoparasiticum* has in a single study been shown to protect crop plants (Davanlou and Hockenhull 1996).

Production of lytic enzymes that degrade host walls may be important in the biological control of plant pathogenic fungi by mycoparasites. For example, Elad et al. (1985) found that the mycoparasite *P. nunn* produced cellulase, glucanase, and chitinase when it grew on cell

walls of host fungi. Deacon and Berry (1992) reviewed the role of mycoparasite derived enzymes in mycoparasitism and indicated the possible involvement of host derived enzymes in the penetration process. In light of this, we have studied the possibility of involvement of enzymes in the penetration process of host hyphae and conidia by *P. mycoparasiticum* and attraction of *P. oligandrum* zoospores to non-diseased and host colonized roots.

Materials and Methods

Fungi used were *Pythium mycoparasiticum* isolates AR5A and AR7A (IMI341972) kindly supplied by J. W. Deacon. *P. oligandrum* isolate MM1 from a Danish soil isolated by the first author. *Fusarium culmorum* isolate IK5 isolated from roots from barley plants in Denmark by I.M.B. Knudsen and *P. ultimum* Trow. var *ultimum* isolate HB2 isolated by H. Wolffhechel from peat.

For enzyme assays *P. mycoparasiticum* isolates were grown in SMV8 [850 ml Modified Czapek-Dox broth (Oxoid, England), 150 ml V8 vegetable juice (Campbell, England) and 4 g calcium carbonate] for 7 days in 250 ml conical flasks at 22 °C. *Pythium ultimum* was grown in 100 ml PDB (Potato Dextrose Broth, Difco, Detroit, MI, USA) for 2 days and *F. culmorum* in PDB for 4 days at 20 °C. Mycelium was harvested, drained twice on ten layers of sterile filter paper, and weighed. *Pythium mycoparasiticum* inoculum was equivalent to 2.0 mg dry mycelium, and inoculum of the host fungi was equivalent to 0.1 g dry mycelium. The two host fungi were used either as living mycelium or lyophilized cell walls. The host fungi were either inoculated separately or simultaneously with *P. mycoparasiticum* in 20 ml SMV8 or, in a few fungal combinations, in 20 ml sterile distilled water in conical flasks (250 ml) at 21 °C. Furthermore, *P. mycoparasiticum* was inoculated to *F. culmorum* conidia (final concentration 1.2×10^6). The experiment was performed once with three repeats of each fungal combination.

Fungal cultures were shaken, samples collected and centrifuged at 11,000 rpm for 8 min. Culture filtrates were used for the enzyme assays. Carboxymethyl-substituted (CM-) and water soluble polysaccharide derivatives, labeled covalently with Remazol Brilliant Blue (RBB) or Remazol Brilliant Violet (RBV) (i.e., CM-cellulose-RBB, CM-Curdlan-RBB and CM-chitin-RBV) were used for the assay of endo-acting cellulase [(endo-1,4 (1,3)-β-D-glucanase)] (EC 3.2.1.4), β-glucanase [(endo-1,3 (1,4)-β-glucanase)] (EC3.2.1.6) and chitinase (EC 3.2.1.14) activity respectively. Gelatin-RBB was used as a substrate for protease i.e trypsin (E.C.3.4.21.4) pronase E (E.C.3.4.24.4) and collagenase. All enzyme substrates were from Loewe Biochimica Blue Substrates, Göttingen, Germany. Assays were performed according to the directions of the manufacturer.

To quantify the activities of the two chitinases, N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) (NAGase) and chitobiosidase (1,4-β-chitobiosidase), the release of p-nitrophenol from p-nitrophenyl N-acetyl-β-D-glucosaminide and p-nitrophenyl β-D-N,N-diacetylchitobiose was estimated. To quantify the cellulytic activities of β-glucosidase (EC 3.2.1.21) and cellobiohydrolase (EC 3.2.1.91) the release of p-nitrophenol from p-nitrophenyl β-D-glucopyranoside or p-nitrophenyl β-D-celloside was estimated. All substrates were from Sigma, USA.

Zoospores were produced as in Madsen et al. (1995). Using methods of Mitchell and Deacon (1986) it was investigated whether *P. oligandrum* zoospores were attracted to the following plant material: tomato (*Lycopersicon esculentum*) seeds and roots, peas (*Pisum sativum*), cress (*Lepidium sativum*), impatiens (*Impatiens wallerana*) and begonia (*Begonia*

elatior). The impatiens and begonia roots were collected from commercial grown plants. Seeds from tomato, peas and cress were germinated on moist filter paper at room temperature. Roots were placed on *F. culmorum* or *P. ultimum* cultures for colonization for two days. The plant material was placed in observation chambers with zoospores and the number of zoospores were counted at different distances from the root tips and compared by χ^2 test.

Results

NAGase activity was detected in co-inoculations of *P. mycoparasiticum* and *F. culmorum* conidia or living *F. culmorum* mycelium (Table 1). However, the conidia themselves had NAGase activity and this activity was as high as the activity recorded in the co-inoculation of *P. mycoparasiticum* isolate AR7A and conidia. A low level of activity was detected when *P. mycoparasiticum* grew in the nutrient solution (Table 1). Endo-chitinase activity was only studied in a minor experiment, and we found that *P. mycoparasiticum* produced extracellular endo-chitinase when it grew in the nutrient solution. In contrast, activity of chitobiosidase was never detected.

P. mycoparasiticum produced extracellular protease both when it grew in the nutrient solution or on hosts and when it starved in water. The activity was significantly higher when *P. mycoparasiticum* was inoculated to host cultures or to the nutrient solution than to water (Table 1). While the protease activity was significantly higher on *P. ultimum* walls than on living *P. ultimum*, no significant difference was detected in inoculations of *P. mycoparasiticum* (AR7A) on walls or living *F. culmorum*. Protease activity was significantly higher on *F. culmorum* walls and living hyphae than on *P. ultimum* walls and hyphae and *F. culmorum* conidia. Furthermore, an isolate variation was seen as isolate AR7A generally produced significantly more protease than AR5A.

β-glucanase activity was induced at day six in co-inoculations of *P. mycoparasiticum* and *P. ultimum* (data not presented) but at day 14 only isolate AR7A induced β-glucanase activity in co-inoculation with *P. ultimum* (Table 1). The two *P. mycoparasiticum* isolates neither excreted β-glucanase in water, on *P. ultimum* walls nor in the nutrient solution (Table 1). *P. ultimum* produced extracellular endo-cellulase, and this activity was reduced when *P. ultimum* was co-inoculated with *P. mycoparasiticum* (Table 2). Low levels of cellobiohydrolase activity were detected when *P. mycoparasiticum* grew on conidia or host walls, but the activities were higher on living *P. ultimum* and *F. culmorum* (Table 2). β-glucosidase activity was detected in all fungal combinations and the activity was higher when *P. mycoparasiticum* was inoculated to living *P. ultimum* than to walls. *Pythium ultimum* did however also excrete extracellular β-glucosidase. β-glucosidase activity was higher when *P. mycoparasiticum* was inoculated to living *P. ultimum* than to living *F. culmorum* but with host hyphae it was the opposite situation. Isolate AR5A produced most β-glucosidase on host walls while there was no significant difference in the production of β-glucosidase by AR7A on living hyphae or walls of *F. culmorum* hyphae (Table 2).

No difference was observed in the number of motile zoospores at different distances from the root tip, indicating that zoospores of *P. oligandrum* were not attracted to host colonized roots nor to non-colonized roots. Furthermore, they were not attracted to tomato seeds colonized by *P. ultimum* nor to non-colonized seeds.

Table 1. Activities[Y] of extracellular NAGase, β-glucanase and protease in cultures of *Pythium mycoparasiticum* isolate AR7A or AR5A and/or living cultures or walls of *Fusarium culmorum* or *P. ultimum* and/or *F. culmorum* conidia.

Mycoparasite host combination	NAGase activity Host alive	β-glucanase activity Host alive	Protease activity Host alive	Host dead
AR7A alone	7.0 ± 0.8	ND[Z]	19.9 ± 1.1	
AR5A alone	5.6 ± 0.6	ND		
AR5A water	3.8 ± 1.0	ND	13.3 ± 0.8	
AR7A plus conidia	10.4 ± 2.2	32. 9 ± 1.0	56.2 ± 1.1	
AR5A plus conidia	18.0 ± 1.4	28.0 ± 2.1	36.9 ± 1.7	
Conidia	12.8 ± 1.3	31.1 ± 3.0		
AR7A plus *F. culmorum*	97.8 ± 2.1		96.5 ± 1.8	99.8 ± 2.0
AR5A plus *F. culmorum*	70.2 ± 1.8		83.1 ± 1.8	50.2 ± 1.2
F. culmorum water	44.4 ± 1.9		ND	91.5 ± 2.2
AR7A plus *P. ultimum*	ND	96.3 ± 3.8	62.5 ± 2.0	29.4 ± 1.8
AR5A plus *P. ultimum*	ND	46.2 ± 4.1	25.9 ± 1.7	
P. ultimum	ND	46.2 ± 4.2	ND	

[Y] The activities are estimated 14 days after inoculation. Fungi were grown in a nutrient solution unless indicated otherwise. Activities are expressed relative to maximum activity of each enzyme and are mean values of three repeats.
[Z] Not detected

Table 2. Activities[Y] of extracellular endo-cellulase, β-glucosidase and cellobiohydrolase in cultures of *P. mycoparasiticum* isolate AR7A or AR5A and/or living cultures or walls of *F. culmorum* or *P. ultimum* or *F. culmorum* conidia.

Mycoparasite host combination	Endo-cellulase activity	β-glucosidase activity		Cellobiohydrolase activity	
	Host alive	Host alive	Host walls	Host alive	Host walls
AR7A alone	ND[Z]			32.4 ± 3.1	
AR5A alone	ND	8.0 ± 1.0		32.0 ± 2.0	
Conidia		9.9 ± 1.4			
AR7A + conidia		11.6 ± 1.0		31.2 ± 1.4	
AR5A + conidia		16.7 ± 1.1		38.5 ± 1.7	
AR7A + *F. culmorum*		25.3 ± 1.4	22.6 ± 1.7	96.7 ± 2.1	39.3 ± 2.0
AR5A + *F. culmorum*		19.2 ± 1.3	26.6 ± 1.9	69.7 ± 2.3	50.8 ± 1.9
F. culmorum water		7.5 ± 1.0			
AR7A + *P. ultimum*	86.7 ± 2.3	98.0 ± 1.9	18.5 ± 1.9	94.2 ± 2.0	32.0 ± 2.2
AR5A + *P. ultimum*	90.9 ± 2.0	46.4 ± 1.6	15.1 ± 0.9		
P. ultimum	97.0 ± 1.8	74.0 ± 2.3		32.9 ± 1.9	

[Y] The activities are estimated 14 d after inoculation. Fungi were grown in a nutrient solution unless indicated otherwise. Activities are expressed relative to maximum activity of each enzyme and are mean values of three repeats.
[Z] Not detected

Discussion

The overall results indicate that mycoparasitism of *P. ultimum* hyphae and hyphae and conidia of *F. culmorum* by *P. mycoparasiticum* did occur in the liquid medium as the enzyme assays showed that enzyme activities generally were higher on host hyphae and conidia than when any of the fungi were inoculated separately (Tables 1 and 2). This is also suggested by the findings that endo-cellulase activity was reduced when *P. mycoparasiticum* was inoculated to *P. ultimum*. *Fusarium culmorum* and *P. ultimum* hyphae growing on agar are earlier described as susceptible to mycoparasitism by *P. mycoparasiticum* (Jones and Deacon 1995, Laing and Deacon 1991). In addition to mycoparasitism, the enzyme assays indicated substrate competition between *P. mycoparasiticum* and living *P. ultimum* in the nutrient solution as the protease activity was significantly higher on *P. ultimum* walls than on living *P. ultimum*. The growth of *P. mycoparasiticum* seems to be higher on *F. culmorum* walls or living *F. culmorum* than on *F. culmorum* conidia because the protease activity was higher in these cultures. This might be due to a slower rate of parasitism of *F. culmorum* conidia than of hyphae, but it might also have been due to different nutritive values of the mycelia and the conidia. The protease activity of *P. mycoparsiticum* was significantly higher on *F. culmorum* than on *P. ultimum*, and this correlates with observations of Jones and Deacon (1995) that *F. culmorum* is a more susceptible host than *P. ultimum*.

Activity of extracellular NAGase in co-inoculations of *P. mycoparasiticum* and living host cultures (Table 1) and the observation that *P. mycoparasiticum* produces endo-chitinase suggest that a break down of chitin in host walls by chitinases might be part of the penetration process. As chitin and protein are parts of the cell walls (Smith and Berry 1974), the proteinases produced by *P. mycoparasiticum* might aid the process of penetration of host hyphae and the growth inside host hyphae. Cellulose and β-glucans are main components of cell walls of oomycetes (Smith and Berry 1974) thus the activity of endo-cellulase, cellobiohydrolase, β-glucosidase and β-glucanase in co-inoculations of *P. mycoparasiticum* and *P. ultimum* might aid the penetration of *P. ultimum* walls. However endo-cellulase and β-glucanase activities were not detected when *P. mycoparasiticum* grew on *P. ultimum* walls and in the nutrient solution hence the activity of endo-cellulase and β-glucanase detected in co-inoculations of *P. mycoparasiticum* and *P. ultimum* might only be produced by *P. ultimum*. Whether *P. ultimum* is protected against its own extracellular enzymes we do not know.

Pythium oligandrum zoospores were not attracted to the tested roots and even though they are able to colonize host-colonized roots (Madsen 1996) they were not attracted to these roots. Likewise, hyphae of both *P. oligandrum* and *P. mycoparasiticum* are not able to orientate their growth towards host hyphae (Laing and Deacon 1991). It is earlier found that *P. oligandrum* zoospores do not accumulate on some roots (A. Pham *personal communication*). Zoospores of plant pathogenic *Pythium* species are attracted to roots of many plants (Mitchell and Deacon 1986). The lack of attraction of *P. oligandrum* zoospores to the tested roots might be a question of testing roots of more different plant species or testing different *P. oligandrum* isolates. We have observed isolate variation in the ability to produce zoospores and oospores (*unpublished data*). It might also be a general feature that mycoparasitic *Pythium* species are not attracted to roots as mycoparasites are physiologically different from plant pathogen *Pythium* species (Foley and Deacon 1986). *Pythium oligandrum* and *P. mycoparasiticum* have many similarities and we are investigating whether this also is true regarding production of the extracellular cellulases, chitinases and proteases which this study indicated were involved in the penetration process of *P. mycoparasiticum*. A quantitative variation in enzyme activity of the two *P. mycoparasiticum* isolates on the different hosts has

been indicated in this study. It will be of interest in connection with biocontrol to know whether this is linked to host specialization in *P. mycoparasiticum*.

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Interaction between tomato and *Pythium oligandrum*: rhizosphere colonization, molecular characterization and induction of defense-related reactions

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Abstract

Pythium oligandrum has received much attention in terms of physiology, ecology, mode of parasitism and potential for the biocontrol of plant pathogens. A recent biological molecular investigation enabled easy differention of *Pythium oligandrum* from other *Pythium* species. *Pythium oligandrum* is also a strong colonizer of the rhizosphere in hydroponic system condition when microflora is nearly absent. In vitro studies have shown that fungal ingress in the root tissues do not induce extensive cell damage and is associated with host cell reactions. Induction of host reactions by *P. oligandrum* is of key importance because it can provide a local protection against pathogenic *Fusarium* spp. This resistance is mainly associated with a strong antagonistic activity in the rhizosphere and in planta as well as with the induction of structural and biochemical barriers that adversely affect pathogen growth and development.

Introduction

Pythium oligandrum Dreschsler is an aggressive mycoparasite of a wide range of fungi including many plant pathogens (Deacon 1976, Drechsler 1943), that has been found to control several seedling diseases under experimental conditions (Al-Hamdani et al. 1983, McQuilken et al. 1990, Vesely 1977, Walther and Gindrat 1987). In epidemiological studies conducted in tomato hydroponic greenhouses, *P. oligandrum* and/or *P. periplocum* were occasionally detected (Rafin and Tirily 1995). Thus, the presence of *P. oligandrum* should be promoted in soilless culture to protect plants against pathogenic attacks. Applied alone or in combination with different potentially useful microorganisms, it may induce a conducted microbial ecology, and consequently, may favor an equilibrium beneficial for plant protection against pathogens either by direct antagonism (mycoparasitism, competition for nutrient) or indirect activity (acquired resistance). Before *P. oligandrum* can be used as a biocontrol agent, questions regarding it's potential utility and it ecology must be addresed. To do this, the objectives of this study were (i) to establish whether *P. oligandrum* can colonize the rhizosphere in commercial greenhouse, (ii) to develop a PCR-RFLP method to quickly characterize *Pythium* spp. in hydroponic system, and (iii) to study *P. oligandrum*-plant interactions, and to detect whether tomato roots inoculated with *P. oligandrum* are protected against pathogenic *Fusarium* spp.

Materials and Methods

Assessment of root colonization by *P. oligandrum* in commercial tomato greenhouses. Mycelial mats bearing many oospores were obtained by growing *P. oligandrum* in liquid culture. Roux bottles (1 liter) containing 200 ml of culture medium were inoculated with three CMA-PARP disks of fungi, then incubated horizontally on one side, usually for 14 days at 25 °C in darkness. The basal liquid culture medium (1.23 g KH_2PO_4, 0.17 g KH_2PO_4, 0.5 g $MgSO_4 \times 7H_2O$, 1.0 mg $FeCl_3 \times 6H_2O$, 30 g cane molasses, 30 mg ergosterol, per liter distilled water) was autoclaved at 121 °C for 15 min. Mycelial mats were then removed, washed in sterile distilled water and fragmented into distilled water using a Waring blender. One week after crop start, tomato roots were first inoculated with a 2×10^3 CFU/ml inoculum of *P. oligandrum* oospores-mycelium in commercial hydroponic system. The second inoculation was done three weeks later with a 6×10^2 CFU/ml inoculum.

Studies on interactions between *P. oligandrum* and plants in situ were as follows. Cytological and ultrastructural investigations to study the relationship between *P. oligandrum* and the plant were conducted following the methods described by Benhamou et al. (1997). Direct isolation of *P. oligandrum* in situ in commercial greenhouse was done by cutting roots into 1 to 2 cm pieces and ten root-pieces were immediately plated on the semi-selective medium CMA-PARP (Jeffers and Martin 1986). Results were expressed as the percentage of the number of root-pieces from which *Pythium* colonies were isolated.

Pythium spp. were characterized by PCR-RFLP. All *Pythium* spp. isolates under study were obtained from tomato roots grown in hydroponic system according to Jeffers and Martin (1986). The mycelium was lyophilized for 24 h and stored at -20 °C until required. DNA was isolated from the mycelial powder using the procedure of Rafin et al. (1995). DNA amplification and electrophoresis in agarose gels was carried out according to a method described elsewhere (Rafin et al. 1995), using the following oligonucleotide primers: PN3 (5' CCgTTggTgAACCAgCggAgggATC 3') and PN34 (5' TTgCCgCTTCACTCgCCg TT 3'). For restriction analysis, each PCR product was digested separately with different restriction enzymes: *AccI*, *AluI*, *HaeIII*, *Hin6I*, *HinfI*, *MspI*, *NdeII*, *RsaI* and *Taq I* (Boehringer Mannheim, France).

Results and Discussion

Colonization by *P. oligandrum* of roots of plants grown in commercial tomato greenhouses was assessed. Six weeks after the first inoculation with *P. oligandrum*, 72% in average of the root segments were colonized. Three weeks later, 63% of the roots segments were still colonized. These results demonstrate that *P. oligandrum* displays the ability to colonize the rhizosphere in a commercial hydroponic system even in the absence of microflora. Previous results showed a weaker rhizosphere colonization when tomato roots were inoculated with *P. oligandrum*, three months instead of one week after the crop start. We assumed that the microflora plays an important role in the rhizosphere colonization by *P. oligandrum*. This fungus must be introduced at the crop start to amplify its effect. However, in further investigations it may be interesting to investigate *P. oligandrum* survival in commercial hydroponic system.

Isolation and characterization of *P. oligandrum*. A CMA-PARP-based, selective medium is an efficient tool to directly detect *Pythium* spp. hyphae on roots, and to distinguish them among other fungi. But, identification of *Pythium* spp. isolates remains often difficult because the reproductive structures needed for diagnosis may lack, or because of highly variable

characteristics within species. Molecular approaches can enable to overcome the limitations of morphological characteristics and provide an efficient tool for a quick and reliable characterization. In this study, PCR and RFLP analysis were used to compare the nuclear genes coding for ribosomal DNA (rDNA) from pure strains of *Pythium* spp. to obtain reference patterns. Two oligonucleotides, PN3 and PN34, were used to amplify the nuclear rDNA region of internal transcribed spacers including the 5.8 S gene. They were complementary to highly conserved regions within the rDNA repeat. The patterns of amplified PCR products and PCR-RFLP for different *Pythium* spp. are presented in Fig. 1.
Amplified PCR products or PCR-RFLP patterns enabled easy differentiation of *P. oligandrum* from other fungi (Fig. 1). For example, the size of *P. oligandrum* PCR product was about 870 bp, instead of 910 bp for *P. ultimum* and 650 bp for *P. uncinulatum*. On the other hand, different restriction enzymes (*Alu I, Hae III*) were required to easily distinguish *P. oligandrum* from *Pythium* group F (Fig. 1).

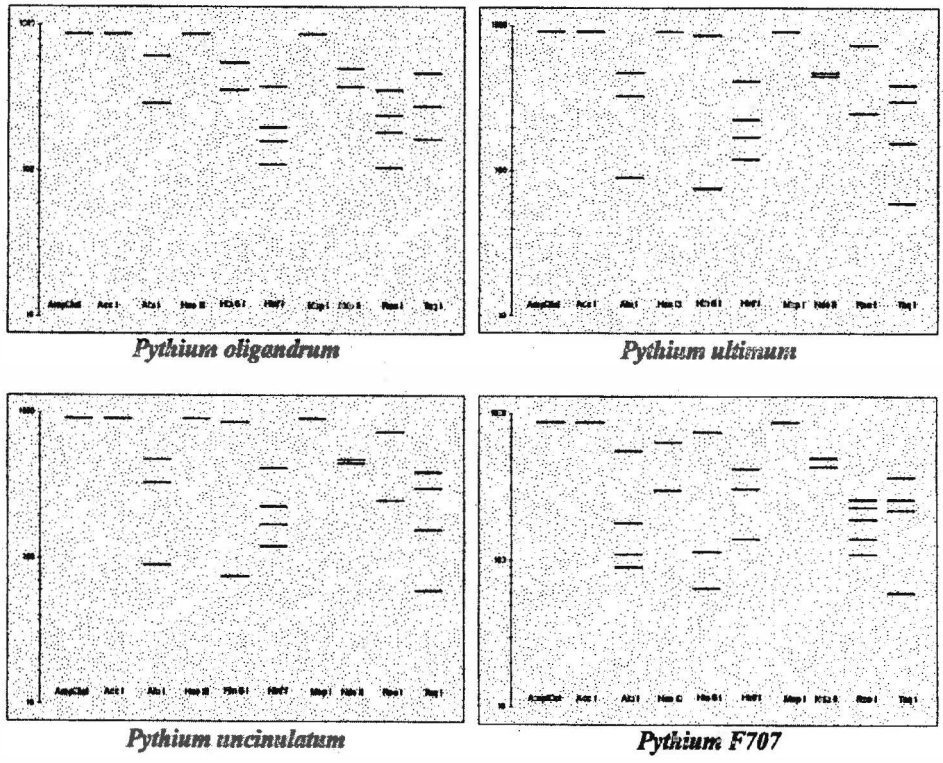

Fig. 1. Patterns of amplified PCR products and DNA restriction fragments with different restriction enzymes of isolates of *Pythium* spp.

Tomato roots were inoculated with the mycoparasite *P. oligandrum* to study the relationship established between the plant and the fungus (Rey 1996). Light microscope investigations of root samples from tomato plants inoculated with *P. oligandrum* revealed that, as early as 48 to 72 h after inoculation, oogonia extensively covered the surface, whereas the epidermis, the cortical and often the vascular stele were usually colonized by hyphae. However, the root system remained free of necrotic symptoms because *P. oligandrum* was unable to cause severe alterations within the colonized root areas. Most invading hyphae (90%) were reduced to empty shells, the only recognizable structure was the fungal wall specifically labeled by exoglucanase-gold complex. A close examination of the invaded areas revealed the occurrence of some host reactions at sites of potential penetration. These host reactions included the formation of different kinds of networks constituted by amorphous material or fibrils encasing the invading hyphae. Wall appositions and papillae were frequently formed at sites of fungal entry. These different host reactions presumably prevent fungal ingress throughout the root tissues. Chemical compounds, likely phenolic derivatives, coated host walls and accumulated inside invaded reacting cells. One may assume that these osmiophilic substances mechanically reinforce the host walls while creating a fungitoxic environment for *P. oligandrum*. Our results demonstrate the ability of *P. oligandrum* to penetrate and grow in tomato root tissues in addition to induce plant defense reactions.

Pythium oligandrum protected tomato roots against *F. oxysporum* f.sp. *radicis-lycopersici*. *Fusarium oxysporum* f.sp. *radicis-lycopersici* hyphae was abundant through much of the epidermis, cortex, endodermis, and paratracheal parenchyma cells in control roots grown in the presence of the pathogen. They rapidly reached the vascular stele and strongly colonized the xylem vessels. In tomato roots inoculated with *P. oligandrum* and challenged with *F. oxysporum* f.sp. *radicis-lycopersici* the pattern of colonization by the pathogen differed markedly. *Fusarium* growth in planta was apparently restricted to the outermost root tissues, including the epidermis and, occasionally, the outermost cortical cells. In contrast, *P. oligandrum* cells were observed in any tissue including the vascular stele. This limited infection by *F. oxysporum* f.sp. *radicis-lycopersici* of tomato roots inoculated with *P. oligandrum* was mainly due to two factors. First, inoculation of tomato roots with *P. oligandrum* induced a number of host reactions that culminated in the formation of structural barriers which likely protected the inner root tissues from pathogen invasion. A typical host reaction was the formation of important wall appositions surrounding the hyphae. *Fusarium oxysporum* f.sp. *radicis-lycopersici* hyphae also met a fungitoxic environnment because most of hyphae were empty. Second, the antagonistic potential expressed by *P. oligandrum* in planta. Observations of numerous sections roots suggested that *P. oligandrum* could interact physically with the pathogen, causing cytological damages. From our ultrastructural studies, we suspect *Pythium* cells to be capable of penetrating and growing within pathogen hyphae. The results obtained demonstrate that tomato plants previously inoculated with *P. oligandrum* gained increased resistance to *F. oxysporum* f.sp. *radicis-lycopersici* attack. This resistance was mainly associated with a strong antagonistic activity in the rhizosphere and in planta, as well as with the induction of structural and biochemical barriers that adversely affected pathogen growth and development.

Pythium oligandrum is a well documented fungus known as a potential biocontrol agent because of its mycoparasitic properties. Surprisingly, little attention has been paid to the relationships established between the plant and this mycoparasite. The present paper demonstrates that *P. oligandrum* can penetrate the root cells without causing cell damage, and induce plant defense-related reactions. We also demonstrate that tomato plants previously

inoculated with *P. oligandrum* are more resistant to *F. oxysporum* f.sp. *radicis-lycopersici* attack. This resistance is mainly associated with a strong antagonistic activity in the rhizosphere and in planta as well as with the induction of structural and biochemical barriers that adversely affect pathogen growth and development (Benhamou et al. 1997). *Pythium oligandrum* can strongly colonize the rhizosphere at the crop start in commercial hydroponic systems. It is easily characterized by PCR-RFLP. *Pythium oligandrum,* along with other microorganisms known to induce plant resistance, e.g., *Penicillium oxalicum* (De cal et al. 1997), plant growth-promoting rhizobacteria (Tuzun et al. 1995), can constitute a key microflora to prevent plants from pathogenic attack. Promoting such a beneficial microflora in soil or hydroponic systems is undoubtedly a promising field of investigation.

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Induced resistance

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Induction and expression of PGPR-mediated induced resistance against pathogens

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Abstract

Treatment of plants with selected strains of plant growth-promoting rhizobacteria (PGPR) can induce systemic resistance in carnation, cucumber, radish, tobacco, and *Arabidopsis* as evidenced by an enhanced defensive capacity upon challenge inoculation with a pathogen. In the induction of resistance by *Pseudomonas* spp. in carnation, radish, and *Arabidopsis*, the O-antigenic side chain of the bacterial outer membrane lipopolysaccharide acts as an inducing determinant, but other bacterial traits are also involved. Siderophores have been implicated in the induction of resistance in tobacco and *Arabidopsis*, and a novel type of siderophore, fluorebactin, may explain induction of resistance associated with salicylic acid (SA) in radish. Although some bacterial strains are equally effective in inducing resistance in different plant species, others show specificity which suggests specific recognition between bacteria and plants at the root surface. Moreover, genetic variation for inducibility by specific PGPR strains is present in carnation and *Arabidopsis*.

In contrast to the phenotypically similar systemic acquired resistance (SAR) induced by pathogens, PGPR-mediated induced systemic resistance (ISR) does not always require SA. SAR-associated SA production induces pathogenesis-related proteins (PRs), but no accumulation of PRs was detectable in radish and *Arabidopsis* expressing ISR. In addition, ISR is fully expressed in *Arabidopsis* plants transformed with the *NahG* gene and unable to accumulate SA. In contrast, *Arabidopsis* mutated in the *Etr1* gene and insensitive to ethylene, or in the *jar1* gene and insensitive to jasmonic acid, were no longer inducible. These results demonstrate that compared to pathogens inducing SAR, non-pathogenic rhizobacteria inducing ISR trigger a different signal-transduction pathway not dependent on the accumulation of SA and activation of PR-genes, but dependent on perception of ethylene and jasmonic acid.

Introduction

Rhizobacteria-mediated induced systemic resistance. Plant growth-promoting rhizobacteria (PGPR) can suppress diseases through antagonism between the bacteria and soilborne pathogens, as well as by inducing a systemic resistance in the plant against both root and foliar pathogens (Schippers 1988). In enhancing the defensive capacity throughout the plant, rhizobacteria seem to activate a signal-transduction pathway leading to the well-studied phenomenon of systemic acquired resistance (SAR) (Hammerschmidt and Kuc 1995, Ryals et al. 1996). However, SAR is induced by limited pathogen infection, avirulent pathogens or cultivar-nonpathogenic races of pathogens causing local necrosis, such as a hypersensitive reaction. PGPR are nonpathogenic and generally considered to not cause harmful effects in the host. SAR is dependent on the production of salicylic acid (SA) in response to infection (Gaffney et al. 1993) and is associated with the accumulation of pathogenesis-related proteins (PRs) both in the inoculated and in distant leaves (Ryals et al. 1996, Van Loon and Van

Kammen 1970). Because exogenous application of SA induces both SAR and PRs, the latter are commonly taken as markers of the induced state.

SAR is non-specific with respect to both the inducing and the challenging pathogen. Thus, a primary infection of cucumber with the fungus *Colletotrichum lagenarium* or with tobacco necrosis virus (TNV) leads to enhanced resistance against various foliar and root diseases caused by fungi, bacteria, and viruses (Kuc 1982). Selected rhizobacterial PGPR strains similarly protected cucumber against anthracnose caused by the fungus *C. orbiculare* (Wei et al. 1991), Fusarium wilt caused by the fungus *Fusarium oxysporum* f.sp. *cucumerinum* (Liu et al. 1995a), bacterial angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* (Liu et al. 1995b), cucurbit wilt disease caused by the bacterium *Erwinia tracheiphila* (Kloepper et al. 1993), and mosaic disease caused by cucumber mosaic virus (Liu et al. 1992). This type of protection was named induced systemic resistance (ISR) as a more general term encompassing SAR (Kloepper et al. 1992).

To conclude that the mechanism responsible for protection is ISR requires that disease suppression be shown to be plant-mediated and that it extends to plant parts that are not in contact with the inducing agent. Accumulation of PRs could additionally be used as an indication that SAR had been induced, although by itself it provides insufficient evidence that induced resistance is the sole mechanism involved. To protect cucumber plants, PGPR strains have been applied by seed treatment, cotyledon injection, or as a soil drench. Under these conditions, the bacteria could invade the plant vascular system and be carried to the aerial parts of the plants (Kluepfel 1993). In the case of protection against Fusarium wilt, it was shown that the inducing bacteria applied to one part of a split root system did not move to the part inoculated with the pathogen (Liu et al. 1995a), indicating protection through ISR. The other studies did not address this question in detail, but it was mentioned that bacteria inducing systemic resistance were not translocated and did not colonize challenged leaves (Liu et al. 1992, 1995b). It cannot be ruled out, however, that antimicrobial metabolites are produced by rhizobacterial strains and transported through the plant in doses sufficient to reduce pathogen activity. In none of these studies were protected plants analyzed for accumulation of PRs, leaving the question whether the protection induced by the PGPR strains was similar to SAR.

Systemic induction of the major PRs of tobacco was associated with ISR against TNV in tobacco treated with the PGPR strain *Pseudomonas fluorescens* CHA0 (Maurhofer et al. 1994). The level of resistance attained was similar to the SAR developing in plants by prior inoculation with TNV. Strain CHA400, a siderophore (pyoverdin)-negative mutant of CHA0, induced partial resistance against the virus, suggesting the involvement of pyoverdin in elicitation of ISR (Table 1). Root colonization of tobacco plants with CHA0 caused an increase in SA in leaves, as did leaf infection with TNV. These results indicate that in tobacco rhizobacteria-mediated ISR and pathogen-induced SAR are both phenotypically and mechanistically similar, with ISR, at least partly, being determined by the siderophore(s) of CHA0. However, the transposon insertion generating the pyoverdin-minus mutation in CHA400 was not localized, and it is not clear whether the loss of pyoverdin is the only mutation in CHA400. Besides pyoverdin, CHA0 produces several metabolites with potentially toxic effects on micro-organisms and plants, including SA (Meyer et al. 1992). Therefore, it is not clear whether the increase in SA in bacterized plants is the result of induction by the bacteria of the synthesis of SA in the plant, or whether the plant takes up bacterial SA and translocates it to the leaves.

Induction of systemic resistance by specific *Pseudomonas* strains has likewise been shown in carnation (Van Peer et al. 1991), radish (Leeman et al. 1995a), *Arabidopsis* (Pieterse et al. 1996) and bean (De Meyer and Höfte 1997). In carnation, *P. fluorescens* strain WCS417 remained confined to the roots and protected plants that were subsequently stem-inoculated with *F. oxysporum* f.sp. *dianthi* from Fusarium wilt. Radish was protected against *Alternaria brassicicola* and different isolates of *F. oxysporum*, as well as *P. syringae* pv. *tomato* (Hoffland et al. 1996). *Arabidopsis* similarly developed ISR against *Peronospora parasitica* (J. Ton *unpublished data*), *F. oxysporum* f.sp.*raphani* and *P. syringae* pv. *tomato* (Pieterse et al. 1996, Van Wees et al. 1997). To demonstrate ISR against root pathogens, a separate inoculation system was developed (Leeman et al. 1995a) in which roots are placed horizontally on rockwool cubes. Adjoining rockwool cubes are compartmentalized through enclosure in plastic bags with only a small incision in the bags allowing protrusion of the radicle into the next compartment. The lower part of the root in one compartment is treated with a bacterial suspension in talcum emulsion. After a few days the upper part of the root in the other compartment is inoculated with the challenge pathogen. In no case were inducing bacteria found to be present in the compartment harboring the challeng pathogen. When the challenger was a leaf pathogen, bacteria were mixed through the soil in which seedlings were planted, and leaves were inoculated at various times afterwards. Induction of resistance against *Botrytis cinerea* was demonstrated in bean upon seed treatment with *P. aeruginosa* strain 7NSK2 (De Meyer and Höfte 1997). Under none of these conditions, were inducing bacteria recovered from the above-ground parts, suggesting that it was highly likely that reduced symptom expression upon challenge with foliar pathogens was due to ISR.

Table 1. Bacterial determinants of induced systemic resistance.

Bacterial strain	Plant species: bacterial determinant	Reference
Pseudomonas aeruginosa		
strain 7NSK2	Tobacco: salicylic acid	De Meyer et al. 1996
	Bean: salicylic acid	De Meyer and Höfte 1997
P. fluorescens		
CHAO	Tobacco: siderophore	Maurhofer et al. 1994
WCS374	Radish: lipopolysaccharide	Leeman et al. 1995b
	siderophore	Leeman et al. 1996
	iron-regulated factor	Leeman et al. 1996
WCS417	Carnation: lipopolysaccharide	Van Peer and Schippers 1992
	Radish: lipopolysaccharide	Leeman et al. 1995b
	iron-regulated factor	Leeman et al. 1996
	Arabidopsis: lipopolysaccharide	Van Wees et al. 1997
P. putida		
WCS358	*Arabidopsis*: lipopolysaccharide	Bakker and Van der Sluis *unpublished data*
	siderophore	Bakker and Van der Sluis *unpublished data*

Bacterial determinants of induced systemic resistance. Of three bacterial strains tested in our group, *P. fluorescens* WCS417 and WCS374 induced systemic resistance in radish against *F. oxysporum* f.sp. *raphani*, whereas, *P. putida* WCS358 did not (Leeman et al. 1995a). All three strains colonized radish roots to the same extent, indicating that both WCS417 and WCS374 possess inducing determinants that are lacking in WCS358. Heat-killed cells were similarly effective and the component responsible was identified as the outer membrane lipopolysaccharide (LPS) (Leeman et al. 1995b) (Table 1). Phage-resistant mutants of WCS417 and WCS374 that lacked the O-antigenic side-chain of the LPS (OA-minus), but colonized radish roots to the same extent as the wild-type strains, were not protective, demonstrating that it is the O-antigenic side-chain of the LPS that was responsible for the induction. Similarly, purified LPS of WCS417 was as effective as live cells in eliciting ISR in carnation (Van Peer and Schippers 1992), establishing the LPS as the main bacterial determinant responsible.

In assays in which radish plants in the separate inoculation system received nutrient solution containing EDDHA as an iron chelator to establish low-iron conditions, possible inducing effects of bacterial siderophores (pseudobactins) were tested. WCS358 and its siderophore-minus mutant did not induce resistance, but WCS374, its siderophore-minus and also its OA-minus mutant did induce resistance. Although the effect was not statistically significant, the same effects were observed for WCS417 and its mutants. Thus, under iron-limited conditions both WCS374 and WCS417 expressed an additional determinant that elicits ISR in radish (Leeman et al. 1996).

Testing the purified pseudobactins of the three strains revealed that the siderophores of WCS358 and WCS417 did not induce resistance, but the one of WCS374 did induce resistance. Even so, the latter was not necessary for the induction by live bacteria, because the WCS374 siderophore-minus mutant was as effective in eliciting ISR as the wild type under low-iron conditions. Therefore, a different iron-regulated metabolite must be responsible, as must also apply to WCS417 (Table 1). Like pseudobactins, SA is produced by several bacteria and acts as an additional iron-chelating siderophore under low-iron conditions (Meyer et al. 1992). Tests for the possible production of SA by the three strains revealed that in vitro at low iron availability strains WCS374 and WCS417 produced approximately 50 and 10 µg SA/ml in standard succinate medium, respectively, whereas, WCS358 did not produce SA. The production of SA by WCS374 and WCS417 decreased concomitantly with the production of pseudobactin when iron availability was increased. SA itself induced resistance in radish at concentrations as low as 100 fg per root tip when applied in talcum emulsion in the separate inoculation system (Leeman et al. 1996). Thus, SA production appeared to fulfill the criteria for the additional bacterial determinant responsible for the induction of systemic resistance under low-iron conditions.

Induction of resistance by *P. aeruginosa* 7NSK2 in bean is likewise iron-regulated (De Meyer and Höfte 1997). Strain 7NSK2 produces three siderophores under iron limitation, pyoverdin, pyochelin, and SA. By using mutants deficient in one or more siderophores, it was demonstrated that SA production was essential for induction of resistance against *B. cinerea*. As SA is itself part of bacterial siderophores of the pyochelin type, a role for this siderophore was not excluded. SA produced by 7NSK2 appeared similarly required for its elicitation of ISR in tobacco against tobacco mosaic virus (De Meyer et al. 1996) (Table 1). In contrast, iron-regulated induction of systemic resistance in cucumber and tobacco by the rhizobacterial strain *Serratia marcescens* 90-166 was not primarily determined by SA, even though strain 90-166 is capable of producing SA in vitro (Press et al. 1997). These observations suggest that

SA-containing siderophores rather than free SA might act as inducers.

To investigate the possible role of SA in the elicitation of ISR in radish under low-iron conditions, the SA-biosynthetic genes from WCS374 were cloned. By mobilizing a gene bank of WCS374 into the WCS358 siderophore-minus mutant, a 28 kb cosmid clone was identified. A 5 kb EcoR1 fragment of this clone was sufficient to convert WCS358, as well as *E. coli*, into SA producers (Mercado-Blanco et al. 1998). This region appeared to be part of a larger operon containing enzymes for the synthesis of an enterobactin type siderophore. Indeed, when the complete 28 kb cosmid clone was transferred into the WCS358 siderophore-minus recipient, a novel siderophore fluorescing blue-green under UV light was produced. This novel siderophore has been designated fluorebactin. Secretion of large quantities of SA by WCS374 in vitro may be an artefact resulting from lack of substrate(s) required for fluorebactin synthesis. If so, synthesis of fluorebactin rather than secretion of SA could explain induction of resistance in radish under low-iron conditions.

Both WCS374 and WCS417, but not WCS358, induced resistance in radish. In contrast, in *Arabidopsis* WCS374 was ineffective, whereas both WCS417 and WCS358 elicited ISR (Van Wees et al. 1997). Apparently, none of the determinants of WCS374 capable of inducing systemic resistance in radish is expressed on, or recognized by *Arabidopsis* roots. Since the O-antigenic side chain of WCS417 acts as the major bacterial determinant in the induction of systemic resistance in carnation and radish, crude cell wall preparations of WCS417 and WCS358 were tested for their capacity to elicit ISR in *Arabidopsis*. Cell walls of both bacterial strains induced systemic resistance (Table 1), but the level attained commonly fell short of that effected by live bacteria (Van Wees et al. 1997). Testing of the OA-minus mutants revealed that these were still inducive, whether tested under low- or under high-iron conditions. However, cell walls of the WCS417 OA-minus mutant no longer induced resistance. These results demonstrated that induction of systemic resistance in *Arabidopsis* by either WCS417 or WCS358 depends on the action of at least two bacterial determinants: the O-antigenic side-chain of the LPS and a metabolite(s) produced by living cells. Like the OA-minus mutant of WCS358, its siderophore-minus mutant still protected *Arabidopsis* against *P. syringae* pv. *tomato*. Moreover, purified pseudobactin of WCS358 induced resistance. These observations are suggestive of iron limitation occurring in the rhizosphere of *Arabidopsis*. Thus, at least for WCS358, both the O-antigenic side-chain of the LPS and the siderophore produced act as factors eliciting ISR (P.A.H.M. Bakker and I. van der Sluis *unpublished data*).

Plant responses to induction of systemic resistance by rhizobacteria. In both pathogen-induced SAR and rhizobacterially-mediated ISR a signal is generated upon induction that is transported systemically throughout the plant, and leads to the state of induced resistance. Although SA can induce both PRs and SAR (Ryals et al. 1996), it is not the translocated signal. Rather SA is required in signal transduction (Vernooij et al. 1994). PRs were associated with the induction of systemic resistance in tobacco by *P. fluorescens* CHA0 (Maurhofer et al. 1994), suggesting that SA might be involved. However, so far this is the only report linking ISR with PRs. No accumulation of proteins cross-reacting with antibodies against PRs was apparent upon induction of ISR in radish by WCS417 (Hoffland et al. 1995) or WCS374 (*unpublished data*), whereas, such accumulation was readily apparent upon induction of SAR by either *P. syringae* pv. *tomato* or SA. Also in *Arabidopsis*, induction of systemic resistance by the rhizobacterial strains was not associated with the accumulation of PRs (Pieterse et al. 1996).

To determine whether SA is required for the establishment of ISR, experiments were conducted using *Arabidopsis* transformed with the *NahG* gene from *P. putida*. The *NahG* gene encodes a salicylate hydroxylase, that converts SA into catechol, preventing SA action. Both development of SAR and accumulation of PRs in response to necrotizing infection are impaired in this transformant (Gaffney et al. 1993). However, WCS417 and WCS358 induced the same extent of protection in untransformed *Arabidopsis* and in the *NahG* transformant, indicating that the induction of systemic resistance by these rhizobacterial strains is independent of SA accumulation (Pieterse et al. 1996, Van Wees et al. 1997). WCS358 is not capable of producing SA and, thus, any ISR dependent on SA would require the plant to produce SA endogenously. However, eliminating the inducing action of SA in the plant still allowed the rhizobacteria to induce resistance. Often, the ISR elicited by rhizobacteria was as strong as pathogen-induced SAR. Hence, whereas, SA is required for the induction of SAR by necrotizing pathogens, it is not required for the phenotypically similar ISR induced by selected strains of non-pathogenic rhizobacteria. Any SA that may be produced by those rhizobacteria likewise does not seem to be involved in generating the induced state.

Within the species *A. thaliana*, differences in inducibility by rhizobacteria were found among ecotypes (Ton et al. 1998, Van Wees et al. 1997). Thus, ecotypes Columbia and Landsberg *erecta* were inducible, as were most other ecotypes tested. In contrast, ecotypes RLD and Ws-O were not. The roots of the latter were colonized to similar extents as those of Columbia and Landsberg *erecta*, suggesting that these ecotypes lack receptors for the bacterial inducing determinants, or are impaired in the subsequent signal-transduction pathway. Both RLD and Ws-O display SAR upon induction with either *P. syringae* pv. *tomato* or SA, indicating that at least the signal-transduction pathway leading to SAR is unimpaired.

Because PRs have been repeatedly suggested to be markers of the induced state (Ryals et al. 1996), and at least some of the PRs have antifungal and antimicrobial activities, PRs have been considered to play a major role in the enhanced defensive capacity of induced plants that is expressed upon challenge inoculation (Van Loon 1997). In spite of repeated attempts, we have been unable to specifically link the induced state in carnation, radish, or *Arabidopsis* to alterations in enzyme activities, inhibitory compounds, electrophoretic protein patterns, or gene expression. However, we recently demonstrated that *Arabidopsis* mutated in the *etr* gene, and as a result insensitive to the plant hormone ethylene (Chang et al. 1993), is no longer inducible by rhizobacteria (Pieterse et al. 1998). Induction of SAR by pathogens or SA occurs normally (Lawton et al. 1995). Using an *Arabidopsis* mutant insensitive to jasmonic acid (JA), *jar1* (Staswick et al. 1992), similar results were obtained (Pieterse et al. 1998). Exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid or of jasmonic acid induced protection against *P. syringae* pv. *tomato* similar to ISR. These observations indicate that our inducing rhizobacteria activate a signal-transduction pathway different from the one leading to SAR, requiring perception of both ethylene and JA rather than SA, even though both pathways result in a phenotypically similar enhanced defensive capacity expressed upon challenge inoculation. Both ethylene and JA are produced by and act as hormones in plants. Our results suggest that recognition of the inducing bacteria by the roots may result in a change in ethylene and JA production or metabolism in the plant.

The availability of bacterial strains that differentially induce ISR and plant ecotypes that are differentially inducible as well as defined *Arabidopsis* mutants with defects in various signal-transduction pathways and defensive activities now offers an excellent starting point for elucidating the physiological, biochemical, and molecular mechanisms involved in PGPR-mediated ISR.

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Genetic analysis of induced systemic resistance in *Arabidopsis thaliana*: association between induced and basal resistance

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Abstract

Selected nonpathogenic rhizobacteria are able to elicit induced systemic resistance (ISR) in plants. Different ecotypes of *Arabidopsis thaliana* were screened for expression of ISR against infection by *Pseudomonas syringae* pv. *tomato*, after treatment of the roots with the nonpathogenic *P. fluorescens* strain WCS417r. From the seven ecotypes tested, two ecotypes (RLD and Ws-0) did not develop ISR after treatment with *P. fluorescens* strain WCS417r. This ISR-nonresponsive phenotype was correlated with a remarkably low level of basal resistance, suggesting that the ability to express ISR is dependent on a threshold level of basal resistance. Subsequently, a cross was made between an ISR-responsive (Col-0) and an ISR-nonresponsive ecotype (RLD). F_1 hybrids were fully capable of expressing ISR and exhibited a relatively high level of basal resistance, indicating that the potential to express ISR and basal resistance are both inherited as dominant traits. Analysis of F_2 plants revealed that both traits segregated in a 3 : 1 fashion in the same set of plants, indicating that the potential to express ISR and basal resistance are monogenically determined and presumably linked.

Introduction

Induced disease resistance is the phenomenon that plants develop an enhanced defensive capacity against a broad spectrum of pathogens upon appropriate stimulation. This resistance response is expressed systemically throughout the plant and is effective against a broad spectrum of pathogens (Hammerschmidt and Kuc 1995). In general, there are two types of induced disease resistance: pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR). SAR is a defense mechanism that is dependent on salicylic acid (SA) and is associated with the accumulation of pathogenesis-related (PR) proteins (Ryals et al. 1996). ISR is a resistance response that is triggered after root treatment with selected strains of nonpathogenic rhizobacteria. Using *Arabidopsis* as a model, it was shown that ISR follows a signalling pathway that, unlike SAR, is independent of SA and PR-gene activation (Pieterse et al. 1996, Van Wees et al. 1997). Apparently, rhizobacteria-mediated ISR and pathogen-induced SAR are controlled by different signalling pathways. However, upon challenge inoculation, SAR and ISR are both expressed as a reduction in disease incidence or severity, suggestive of a similar defensive mechanism becoming activated. After infection by a pathogen, SAR-induced plants are characterized by a rapid and intense activation of a broad spectrum of defense mechanisms. So far, cell wall lignification (Hammerschmidt and Kuc 1982), increased peroxidase activity (Simons and Ross 1970, Van Loon 1976, Ye et al. 1990), accumulation of hydroxyproline-rich proteins (Hammerschmidt et al. 1984), callose deposition (Ye et al. 1991), and accumulation of antifungal PR-proteins (Van Loon 1985) have been reported as possible mechanisms contributing to the enhanced resistance state of SAR. In contrast, not much is known about the

defense mechanisms that are activated during rhizobacteria-mediated ISR.

The main objective of this study was to elucidate the genetic basis of rhizobacteria-mediated ISR and to investigate the relationship between ISR and basal resistance in *Arabidopsis*. Based on the observation of Van Wees et al. (1997) that the *Arabidopsis* ecotype RLD fails to express ISR after treatment with *P. fluorescens* strain WCS417r, we started a classic genetic approach. Several *Arabidopsis* ecotypes were screened for their potential to express WCS417r-mediated ISR against the leaf pathogen *P. syringae* pv. *tomato*. Subsequently, crosses were made between WCS417r-responsive and WCS417r-nonresponsive ecotypes. In this study, we demonstrate that the WCS417r-nonresponsive phenotype of two *Arabidopsis* ecotypes correlates with a remarkably low level of basal resistance against *P. syringae* pv. *tomato*. Furthermore, we provide evidence that the potential to express WCS417r-mediated ISR and basal resistance against *P. syringae* pv. *tomato* are both inherited as a single dominant trait.

Materials and Methods

The rifampicin-resistant *P. fluorescens* strain WCS417r (Van Peer et al. 1991), was grown on King's medium B agar plates (King et al. 1954) for 24 h at 28 °C. The virulent bacterial leaf pathogen *P. syringae* pv. *tomato* strain DC3000 and the avirulent strain *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) (Whalen et al. 1991) were cultured overnight in liquid King's medium B at 28 °C. The bacterial cells were collected and resuspended in 10 mM $MgSO_4$. Seedlings of *A. thaliana* ecotypes Col-0, Ler, Cvi, Shah, Kas, RLD, and Ws-0, and F_1 plants of the Col-0 x RLD cross were grown in quartz sand for two weeks. Subsequently they were transferred to pots (60 ml), containing a sand/potting soil mixture that had been autoclaved twice before application of WCS417r or 10 mM $MgSO_4$. Plants were cultivated in a growth chamber with a 9-h day (200 mE/m^2.sec at 24 °C) and 15-h night (20 °C) cycle and 65% relative humidity. Plants were watered on alternate days and once a week supplied with nutrient solution.

The *Arabidopsis-P. syringae* bioassay. Prior to transfer of the *Arabidopsis* seedlings to the pots, a suspension of ISR-inducing WCS417r bacteria (10^9 CFU/ml) was mixed thoroughly through the sterile sand/potting soil mixture to a final density of 5 x 10^7 CFU/g. Control soil was supplemented with an equal volume of sterile 10 mM $MgSO_4$. SAR induction was performed 4 days before challenge inoculation by pressure-infiltrating two lower leaves with a suspension of *P. syringae* pv. *tomato* (*avrRpt2*) at 10^7 CFU/ml in 10 mM $MgSO_4$. One day before challenge inoculation, the plants were placed at 100% relative humidity. Five-week-old plants were inoculated by dipping the leaves in a *P. syringae* pv. *tomato* suspension containing 2.5 x 10^7 CFU/ml in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). Four or five days after challenge inoculation, the proportion of leaves with symptoms was determined per plant (n = 20-25). Data were statistically analyzed using Student's t-test or an analysis of variance followed by the Fisher's LSD test.

For quantification of basal resistance, leaves of 5-week-old plants were inoculated by pressure infiltrating a suspension of *P. syringae* pv. *tomato* at 5 x 10^5 CFU/ml in 10 mM $MgSO_4$. Two days after inoculation, five leaf samples per ecotype were collected, weighed, and homogenized in 10 mM $MgSO_4$. Subsequently, dilutions were plated onto selective King's medium B agar supplemented with 50 mg/L rifampicin and 100 mg/L cycloheximide. After incubation for 48 h at 28 °C, the number of rifampicin-resistant CFU per g of infected leaf tissue was determined.

Results

Different *Arabidopsis* ecotypes were tested for their potential to express WCS417r-mediated ISR and pathogen-induced SAR against *P. syringae* pv. *tomato* infection. In all ecotypes tested, predisposing infection with the avirulent pathogen *P. syringae* pv. *tomato* (*avrRpt2*) resulted in the development of SAR against infection by the virulent strain of *P. syringae* pv. *tomato* (Table 1). WCS417r-mediated ISR was not expressed in ecotypes RLD and Ws-0, in contrast to the other ecotypes, which showed significant disease reduction after WCS417r treatment (Table 1). In order to investigate the relationship between the potential to express ISR on the one hand, and basal resistance on the other hand, growth of *P. syringae* pv. *tomato* in leaves of non-induced plants was determined for all ecotypes. Interestingly, the WCS417r-nonresponsive ecotypes RLD and Ws-0 exhibited a significantly faster growth of *P. syringae* pv. *tomato* than the responsive ecotypes (Table 1), indicating that ecotypes RLD and Ws-0 have a lower level of basal resistance against *P. syringae* pv. *tomato* than the WCS417r-responsive ecotypes.

Table 1. Quantification of induced resistance and basal resistance against *P. syringae* pv. *tomato* in different ecotypes of *A. thaliana* [X].

Ecotype	Control[Y]	Test for ISR[Y]	Test for SAR[Y]	Test for basal resistance (x 10^9)[Z]	
Col-0	100a	58b	38c	0.04	± 0.01
Ler	100a	54b	42c	0.21	± 0.21
Cvi	100a	81b	62c	3.30	± 0.95
Shah	100a	68b	57c	3.82	± 1.81
Kas	100a	83b	55c	4.67	± 0.99
RLD	100a	105a	37b	11.30	± 2.25
Ws-0	100a	105a	57b	12.60	± 4.83

[X] The bioassays for the quantification of induced resistance and basal resistance were performed as described in Materials and Methods.

[Y] Numbers are the proportion of leaves with symptoms relative to the control treatment (100%) at 4 days after challenge inoculation. Different letters in the same row indicate statistically significant differences (Fisher's LSD test, P=0.05).

[Z] Numbers are means ± SEM of the amount of CFU of *P. syringae* pv. *tomato* per g fresh weight, 2 days after inoculation.

Expression of ISR and basal resistance are inherited as single dominant traits. To elucidate the genetic basis underlying the potential of a plant to express ISR, the WCS417r-responsive ecotype Col-0 was crossed with the WCS417r-nonresponsive ecotype RLD. Subsequently, F_1 plants were tested for their ability to express WCS417r-mediated ISR. WCS417r-treated F_1 plants developed a similar level of protection against *P. syringae* pv. *tomato* infection as the Col-0 parent (Table 2), indicating that responsiveness to WCS417r is inherited as a dominant trait. Similarly, the level of *P. syringae* pv. *tomato* infection in control-treated F_1 plants was comparable with that of the control-treated Col-0 parent (Table 2), indicating that basal resistance against *P. syringae* pv. *tomato* infection is inherited as a dominant trait as well.

To investigate whether the potential to express ISR and basal resistance are monogenic or multigenic traits, F_2 plants of the Col-0 x RLD hybrids were analysed. Approximately 25% of the F_2 plants (11 out of 47) were nonresponsive to WCS417r-treatment and exhibited a disease severity level comparable with RLD plants. Approximately 75% of the F_2 plants (36 out of 47) were responsive to WCS417r treatment and exhibited a disease severity level comparable with WCS417r-treated Col-0 plants. The observation that basal resistance and the potential to express ISR follow a 3 : 1 segregation in the same set of plants, suggests that both traits are monogenically determined and linked.

Table 2. Quantification of induced resistance and basal resistance against *P. syringae* pv. *tomato* in Col-0, RLD and F_1 hybrids of the Col-0 x RLD cross[Y].

	Control treatment[Z]	WCS417r treatment[Z]
Col-0	100a	36b
RLD	184a	210a
F_1	92a	46b

[Y] The bioassays for the quantification of induced resistance was performed as described in Materials and Methods. The level of basal resistance can be deduced from the disease severity observed in control-treated plants.

[Z] Numbers are the proportion of leaves with symptoms relative to the control treatment of Col-0 (100%) at 4 days after challenge inoculation. Different letters in the same row indicate statistically significant differences compared to the control treatment (Student's t-test, P=0.05).

Discussion

Rhizobacteria-mediated ISR and pathogen-induced SAR are two plant resistance responses that are controlled by different signalling pathways (Pieterse et al. 1996, Van Wees et al. 1997). The observation that pathogen-induced SAR can be expressed in the *Arabidopsis* ecotypes RLD and Ws-0, whereas WCS417r-mediated ISR is not (Table 1), provides additional evidence that ISR and SAR are different plant responses. However, the cause of the WCS417r-nonresponsive phenotype of RLD and Ws-0 remains to be clarified. RLD and Ws-0 might be affected in the recognition of the ISR-inducing rhizobacteria, or alternatively, be disturbed in the signalling pathway controlling ISR. Another explanation could be that expression of ISR in RLD and Ws-0 is dependent on the level of basal resistance. The observation that RLD and Ws-0 have a remarkably low level of basal resistance against *P. syringae* pv. *tomato* (Table 1), suggests that the potential of a plant to express ISR requires resistance mechanisms to be sufficiently active against the challenging pathogen. We demonstrated that basal resistance and the potential to express ISR are both inherited as a dominant trait in F_1 plants of the Col-0 x RLD cross (Table 2). Furthermore, we showed that basal resistance and potential to express ISR both follow a 3 : 1 segregation in the same set of F_2 plants, indicating that both traits are monogenically inherited and presumably linked. The results support the notion that ISR is an enhancement of extant resistance (Van Loon 1983). RLD and Ws-0 appear to lack the ability to express ISR against *P. syringae* pv. *tomato* because they possess only a low level of basal resistance against this pathogen.

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Induction of systemic resistance by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 is a salicylic acid dependent phenomenon in tobacco

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Abstract

Colonization of plant roots by rhizobacteria can activate a resistant state throughout the whole plant. The induced resistance is similar to systemic acquired resistance (SAR) that appears after pathogen infection. It is currently unclear if systemic resistance induced by the rhizobacteria involves the same signalling pathway as SAR induced by pathogens. In this study we demonstrated that, in the tobacco-tobacco mosaic virus (TMV) model, resistance induced by *Pseudomonas aeruginosa* 7NSK2 was similar to SAR induced by TMV. Treatment with both inducers resulted in a similar resistance in wild type tobacco and *P. aeruginosa* 7NSK2 did not induce resistance in NahG plants. Along with the essential role for SA produced by *P. aeruginosa* 7NSK2 in SAR, these results suggest that *P. aeruginosa* 7NSK2 treatment activates the SAR pathway.

Introduction

After a prior infection with a necrotizing pathogen plants generally acquire a systemic and broad spectrum disease resistance. This systemic acquired resistance (SAR) is associated with an induction of pathogenesis-related (PR) proteins (Ward et al. 1991) and with an increase in salicylic acid (SA) levels (Shulaev et al. 1995). SA accumulation is a determining factor for SAR expression because Vernooij et al. (1994) could not demonstrate SAR in transgenic plants that degrade SA (NahG plants). If SA is also the signal responsible for the systemic activation of SAR remains an open question. SA transport from the SAR induction site to systemic leaves was associated with the induced resistant state (Shulaev et al. 1995), but was not the only determining factor for the systemic induction of resistance since SAR could be induced from NahG leaves (Vernooij et al. 1994).

Plant growth-promoting rhizobacteria (PGPR) can also induce a broad spectrum disease resistance in several host plants (Pieterse et al. 1996b, Tuzun and Kloepper 1995). This phenomenon has been termed induced systemic resistance (ISR) because it is currently unclear if it involves the same signalling as SAR.

Pseudomonas aeruginosa 7NSK2 is a PGPR (Höfte et al. 1994) that additionally controlled Pythium damping-off in tomato (Buysens et al. 1996). To acquire iron in an iron-limited environment, such as plant roots, this strain produces three types of siderophores, pyoverdine, pyochelin, and SA (Buysens et al. 1996). In bean, *P. aeruginosa* 7NSK2 induced systemic resistance to *Botrytis cinerea* and *Colletotrichum lindemuthianum* (Bigirimana et al. 1997). In this study we used the tobacco mosaic virus (TMV)-tobacco model and its molecular tools to investigate if ISR induced by *P. aeruginosa* 7NSK2 is similar to classical SAR.

Materials and Methods

Bacterial strains. Siderophore-deficient mutants of *P. aeruginosa* 7NSK2 used in this study are represented in Table 1. Bacteria were grown overnight on King's medium B (King et al. 1954) agar at 37 °C and suspended in sterile demineralized water for inoculation. Root colonization by the bacteria was checked at the end of every experiment as previously described (De Meyer and Höfte 1997).

Table 1. *Pseudomonas aeruginosa* 7NSK2 and derived mutants

Strain/mutant	Siderophore production[z]	Reference
7NSK2	pvd^+, pch^+, SA^+	Iswandi et al. (1987)
MPFM1	pvd^-, pch^+, SA^+	Höfte et al. (1991)
KMPCH	pvd^-, pch^-, SA^+	Höfte et al. (1993)
7NSK2-562	pvd^+, pch^-, SA^-	De Meyer and Höfte (1997)
MPFM1-569	pvd^-, pch^-, SA^-	De Meyer and Höfte (1997)

[z]pvd = pyoverdine, pch = pyochelin, SA = salicylic acid

Tobacco assay for induced systemic resistance to TMV. PGPR-treated plants were compared to non-treated plants (disease control) and to plants where systemic resistance was induced by a TMV pre-inoculation of one (TMV1) or three (TMV3) lower leaves (Ross 1961). Four week-old tobacco seedlings (*Nicotiana tabacum* cv. Samsun NN or NahG-10) (Gaffney et al. 1993) were transferred to pots with 450 g of compost potting soil (Klassmann Deilmann, Geeste, Germany) mixed with bacteria to a concentration of about 5 x 10^7 CFU/g soil. Seedling roots were soaked for 5 min in a bacterial suspension of 10^9 CFU/ml prior to planting. Plants were grown in the greenhouse at 24 ± 5 °C. Four and six weeks after transfer, 10 ml of a bacterial solution containing 10^7 CFU/ml was watered to each plant. Six weeks after transfer, one or three lower leaves were inoculated with TMV to induce SAR. One week later one leaf of all plants was challenged with TMV. Induced resistance was assessed 5 or 7 days after challenge for NahG-10 and Samsun NN plants, respectively, by measuring the diameter of ten randomly chosen TMV lesions per leaf. Treatments consisted of at least eight replicates. Statistical analysis was performed by analysis of variance, and treatment means were compared with Fisher's least significant difference (LSD) test at $P = 0.05$.

RNA extraction and RNA gel blot analysis. In Samsun NN plants, accumulation of *PR-1a* mRNA was checked on non-challenged leaves 7 and 9 days after the induction treatment. Total RNA was extracted with guanidine hydrochloride (Logemann et al. 1987) and 10 µg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond-N^+ membranes (Amersham, Gent, Belgium) by capillary transfer (Sambrook et al. 1989). Blots were hybridized at 42 °C with a DIG-labelled *PR-1a* cDNA probe (Payne et al. 1988) and detection was performed with CSPD (DIG DNA labelling and detection kit, Boehringer-Mannheim, Brussels, Belgium).

Results

In Samsun NN plants, systemic resistance to TMV is only induced by *P. aeruginosa* 7NSK2, MPFM1 and KMPCH, strains that produce SA (Table 2). Mutants deficient in SA production like 7NSK2-562 and MPFM1-569 did not induce resistance. On average, the induced resistance had a strength that was intermediate between SAR induction on one and three lower leaves with TMV. The potential level of systemic resistance induced by *P. aeruginosa* 7NSK2 is probably comparable to that of a TMV pre-inoculation on three lower leaves, because in all experiments that level of resistance was observed in some plants of the 7NSK2, MPFM1 or KMPCH treatment. The bacterial induced resistance showed, however, a clearly higher variability. It is unlikely that this variability is caused by differences in root colonization because these did not correlate with differences in induced resistance. Induction of systemic resistance by *P. aeruginosa* 7NSK2 was not observed in NahG-10 tobacco (Table 2).

Table 2. Mean TMV lesion diameter upon TMV infection of tobacco plants treated with *P. aeruginosa* 7NSK2 and derived mutants or pre-treated with TMV on lower leaves. Statistical analysis was performed by analysis of variance and means were compared with Fisher's LSD test at $P = 0.05$.

	Samsun NN			NahG-10	
Treatment	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2
control	3.9 b	5.3 a	3.8 a	5.1 a	5.0 a
TMV1	2.7 c	3.3 cd	2.5 b	-	-
TMV3	1.6 e	3.1 d	1.9 c	-	-
7NSK2	2.4 cd	4.6 b	2.2 bc	4.7 a	4.7 a
MPFM1	1.9 de	3.7 c	-	-	-
KMPCH	2.1 d	3.8 c	2.0 c	4.6 a	5.0 a
7NSK2-562	4.3 ab	5.4 a	-	-	-
MPFM1-569	4.5 a	5.3 a	3.7 a	4.8	4.7 a
LSD	0.5	0.6	0.4	0.6	0.5

Up to now a systemic induction of *PR-1a* mRNA has not been detected in leaves of *P. aeruginosa* 7NSK2-treated plants. This is probably a technical problem, since the previously demonstrated *PR-1a* mRNA accumulation in systemic leaves of the TMV3 treatment (Ward et al. 1991) was not detected either.

Discussion

This study shows that SA production by *P. aeruginosa* 7NSK2 is not only important for induced systemic resistance in bean (De Meyer and Höfte 1997), but also for induced resistance to TMV in Samsun NN tobacco because only SA-producing strains induced resistance to TMV (Table 2). Iron-regulated compounds are also involved in ISR by other rhizobacteria such as *P. fluorescens* CHA0 (Maurhofer et al. 1994), *P. fluorescens* WCS374 and WCS417 (Leeman et al. 1996) and *Serratia marcescens* 90-166 (Press et al. 1997). All strains mentioned produce SA, but for none of them conclusive evidence for SA involvement in induced resistance has been found. On the contrary, SA-deficient mutants of *S. marcescens* 90-166 still induced resistance, indicating that SA was not the sole determinant for ISR (Press et al. 1997). This does not totally exclude a role for SA since another iron-regulated

compound might have served as alternative induced resistance inducer in the SA-deficient mutants. This is not mere hypothesis since such a two compound, mutually exchangeable, ISR induction has been demonstrated for *P. fluorescens* WCS374 and WCS417. Cell wall lipopolysaccharides and an iron-regulated compound were bacterial determinants and the induction of systemic resistance and this could only be repressed when both determinants were eliminated (Leeman et al. 1996).

Because *S. marcescens* 90-166 and *P. fluorescens* WCS417 still induced systemic resistance in NahG plants (Pieterse et al. 1996a, Press et al. 1997) at least one of their ISR-inducing pathways might differ from those activated in case of classical SAR. Because the SA dependent induction of resistance by *P. aeruginosa* 7NSK2 could not be demonstrated in NahG plants (Table 2), this strain does not seem to induce an alternative pathway for induction of systemic resistance. The role for bacterial and/or plant SA in planta even strongly suggests that *P. aeruginosa* 7NSK2 activates SAR. This statement needs to be confirmed by a systemic accumulation of PR proteins.

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Induced resistance against Fusarium wilt of tomato by *Penicillium oxalicum* is not associated to pathogenesis-related proteins

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Abstract

Penicillium oxalicum has been shown to induce resistance in tomato against *Fusarium oxysporum* f.sp. *lycopersici*. Plants were either previously treated in the seedbed with a conidial suspension (10^7 conidia/ml) of *P. oxalicum* seven days before transplanting or were soaked in a *P. oxalicum* conidia solution 30 min before transplanting. *F. oxysporum* f.sp. *lycopersici* race 2 was inoculated just before transplanting. Disease severity and stunting caused by *F. oxysporum* f.sp. *lycopersici* were significantly reduced ($P = 0.05$) in tomato plants treated with *P. oxalicum* in a 45% and 25%, respectively. No changes in protein patterns and no accumulation in the pathogenesis-related proteins (PRs) in tomato plants treated with *P. oxalicum* were observed. The induction of mRNA of extracellular 35kDa acidic β-1,3-glucanase and intracellular 33 kDa basic β-1,3-glucanase by *F. oxysporum* f.sp. *lycopersici* was recorded. Association between induced resistance and PRs accumulation is discussed in our system.

Introduction

Induced resistance can be a tool to integrate into a management program to control *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) Snyder & H.N. Hansen, the causal agent of Fusarium wilt of tomato. Resistance against *F. oxysporum* f.sp. *lycopersici* in tomato plants has been obtained using non-pathogenic or other formae speciales of *F. oxysporum* (Tamietti et al. 1993), other microorganisms (Verma and Allison 1970), or abiotic factors (Abbattista et al. 1988). De Cal et al. (1997a) reported that *Penicillium oxalicum* Currie & Thom induced resistance on tomato plants to *F. oxysporum* f.sp. *lycopersici*. Many compounds have been described in tomato plants which may be responsible for enhanced resistance to *F. oxysporum* f.sp. *lycopersici*: an increase in the levels of peroxidase and polyphenoloxidase activities (Abbattista et al. 1988), β-1,3-glucanase and chitinase activities (Tamietti et al. 1993), phenols (Abbattista et al. 1988), and changes in calcium levels (Beckman et al. 1991).

Results of this work suggest that the observed induction of resistance by *P. oxalicum* in tomato plants is not associated to the induction of novel proteins.

Materials and Methods

Isolates of *Fusarium oxysporum* f.sp. *lycopersici* (race 2) and *Penicillium oxalicum* were maintained and grown as described by De Cal et al. (1995).

The tomato (*Lycopersicum esculentum* Mill.) cultivar Lorena (S&G, Sandoz Seeds), susceptible to race 2 of *F. oxysporum* f.sp. *lycopersici* and resistant to race 1, were used. Tomato seeds were sown in trays containing an autoclaved mixture of vermiculite-peat (1 : 1,

v : v) and maintained in a chamber at 22 to 28 °C with fluorescent light (100 µE /m^2/s, 16 h photoperiod) and 80 to 100% humidity for about 3 weeks.

Samples (from roots, stems and leaves, independently) were ground in liquid nitrogen. Three ml/g f.w. of acidic (0.1 M sodium citrate-phosphate, 15 mM mercaptoethanol, pH 2.8) or neutral (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) buffer was added and the suspension was centrifuged at 12,000 *g* for 20 min at 0 °C. The extract was filtered through a PD10 desalting-column (Pharmacia) and precipitated with 80% acetone. Protein extract enriched in cell wall proteins was obtained as described by Molina et al. (1993). Proteins were fractionated in a SDS polyacrylamide either 15% or 4 to 20% gradient gel electrophoresis following the manufacturer's instructions (Bio-Rad).

Standard molecular techniques employed in this study (RNA purification, Northern blot and hybridization, agarose gel electrophoresis) were performed as described by Sambrook et al. (1989) and following manufacturing indications (Amersham)

Results

Twenty four day-old seedling plants were soaked in a *P. oxalicum* conidial suspension (10^7 conidia/ml) for 30 min before being transplanted to 15 x 15 cm pots containing peat (15 plants per pot). The first sample was collected 20 min after transplanting. Six more samples were collected after 1, 2, 3, 4, 7 and 14 days. Proteins were extracted with a neutral and acidic buffer and fractionated in 15% acrylamide SDS-page. Results indicated that there were no differences in the protein patterns of extracts obtained with the different treatments (*data not shown*).

Tomato seedlings in seedbeds were treated 7 days before transplanting with a conidial suspension of *P. oxalicum* (10^7 conidia/ml; 60 ml were applied per liter substrate). Seedlings from seedbeds with two true leaves were transplanted into flasks containing sterile Hoagland no. 2 solution as described in De Cal et al. (1997b). Flasks were infested with a suspension of 10^4 microconidia/ml of *F. oxysporum* f.sp. *lycopersici* in water just before transplanting. Control treatments were: (i) plants infected with *F. oxysporum* f.sp. *lycopersici* and untreated with *P. oxalicum* (ii) plants uninfected with *F. oxysporum* f.sp. *lycopersici* and treated with *P. oxalicum* and (iii) plants uninfected with *F. oxysporum* f.sp. *lycopersici* and untreated with *P. oxalicum*. Five replicate flasks, each containing four plants, were used per treatment. The flasks were placed in a randomized block design in a growth chamber. Disease severity and reduction in plant size induced by *F. oxysporum* f.sp. *lycopersici* were calculated as described in De Cal et al. (1995) 11 days after transplanting. A 45% reduction in disease severity and a 25% reduction in stunting were observed ($P = 0.05$) in tomato plants treated with *P. oxalicum*. Plants were then harvested and stems, roots and leaves were stored, separately, at -80 °C. Proteins were extracted with a neutral or acidic buffer. Also cell wall enriched protein extract was obtained. All samples were fractionated in 4 to 20% gradient gel acrylamide SDS-page. SDS-PAGE patterns from proteins extracted from roots, stems and leaves of tomato plants (untreated, *P. oxalicum*-treated, *P. oxalicum*-untreated and *F. oxysporum* f.sp. *lycopersici*-inoculated, *P. oxalicum*-treated and *F. oxysporum* f.sp. *lycopersici*-inoculated) did not show differences with any extraction method used (neutral, acidic or cell wall extraction) (Fig. 1).

Total RNA was extracted from leaves of *P. oxalicum*-untreated and *F. oxysporum* f.sp. *lycopersici*-uninoculated, *P. oxalicum*-treated and *F. oxysporum* f.sp. *lycopersici*-uninoculated, *P. oxalicum*-untreated and *F. oxysporum* f.sp. *lycopersici*-inoculated, *P. oxalicum*-treated and *F. oxysporum* f.sp. *lycopersici*-inoculated tomato plants. Northern

blot hybridization was used to investigate the expression of selected pathogenesis-related genes (PRs) using homologue probes specifically induced in a plant-fungus interaction. DNA probes were kindly provided by Prof. P. deWit (University of Wageningen. The Netherlands) and were those encoding an acidic chitinase (Chi3), basic chitinase (Chi9), acidic β-1,3-glucanase (Gluac), basic β-1,3-glucanase (Glubas) and the PR1 protein of tomato (Danhash et al. 1993, Van Kan et al. 1992).

Fusarium oxysporum f.sp. *lycopersici* induced basic and acidic glucanases (Fig. 2). Basic glucanases had a considerable level of basal expression. Chi3 hybridized with all extracts (*data not shown*). Other probes, Chi9 and PR-1, did not hybridize with any extract. No differential hybridization was observed in *P. oxalicum*-treated extracts.

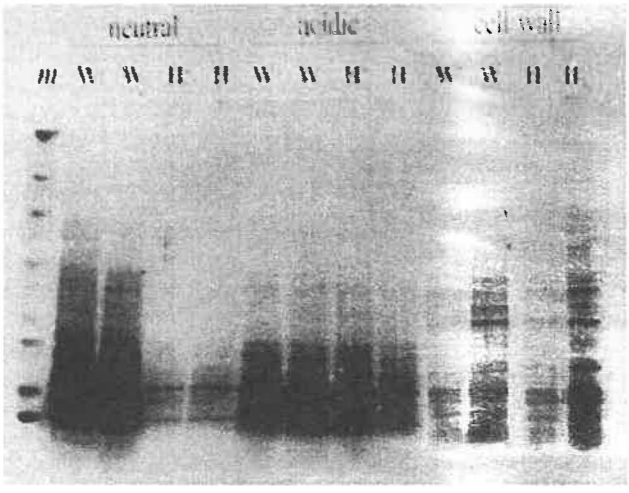

Fig. 1. Acrylamide gradient SDS-PAGE 4 to 20% of tomato plant leaves extracted with neutral, acidic or LiCl (enriched with cell wall proteins) buffers. *m* = low molecular weight markers (Bio-Rad); Protein extracts from: *F. oxysporum* f.sp. *lycopersici*-inoculated plants (W); *P. oxalicum*-treated and *F. oxysporum* f.sp. *lycopersici*-inoculated plants (W^+); *P. oxalicum*-treated plants (H^+) and non-treated and non-inoculated plants (H).

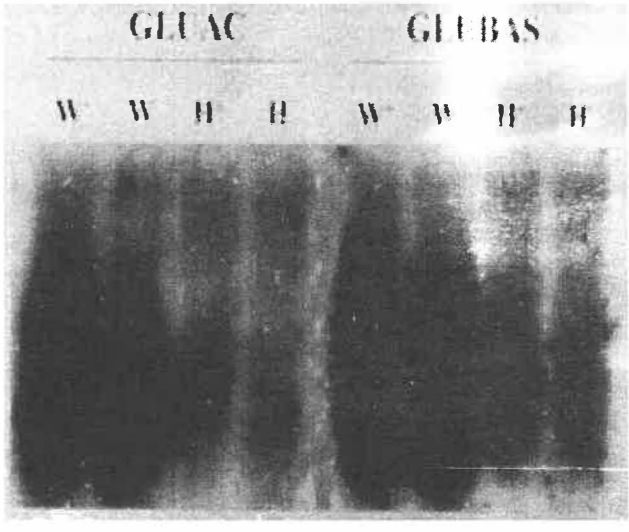

Fig. 2. Northern blot analysis of total RNA extracted from tomato plant leaves hybridized with acidic β-1,3-glucanase (GLUAC) and basic β-1,3-glucanase (GLUBAS) probes. RNA extracts from: *P. oxalicum*-treated and *F. oxysporum* f.sp. *lycopersici*-inoculated plants (W^+); *F. oxysporum* f.sp. *lycopersici*-inoculated plants (W); *P. oxalicum*-treated plants (H^+) and non-treated and non-inoculated plants (H).

Discussion

P. oxalicum treatment significantly reduced severity of disease and stunting caused by *F. oxysporum* f.sp. *lycopersici* in tomato plants. Results presented here showed no changes in protein patterns and no accumulation of the PRs assayed in tomato plants treated with *P. oxalicum*. These results suggest that defense mechanisms in our system are not related to accumulation of PRs. Other authors have described similar results (Hoffland et al. 1995). However, there are several reports relating induced resistance with a concomitant accumulation of PR proteins (Hammerschmidt and Ku• 1995).

We demonstrated here for the first time the induction of mRNA of extracellular 35 kDa acidic β-1,3-glucanase and intracellular 33kDa basic β-1,3-glucanase by *F. oxysporum* f.sp. *lycopersici* (Fig. 2). These mRNA PR proteins accumulation were described previously in the interaction of tomato plants and *Cladosporium fulvum* (Van Kan et al. 1992). The accumulation of PR glucanases in infested tomato plants may be part of a general defense response of plants against pathogens. Wubben et al. (1993) observed that upon infection by *C. fulvum*, tomato plants started to produce PR proteins (glucanase, chitinase and PR-1b) near the stomata in *C. fulvum*-inoculated tomato leaves. Van den Elzen et al. (1993) also found that tomato plants constitutively expressing both class I β-1,3-glucanase and class I chitinase genes exhibit enhanced resistance against *F. oxysporum* f.sp. *lycopersici*.

Our results suggest that *P. oxalicum*-induction of resistance is possible in tomato plants in which no further accumulation of PR proteins occurs. However, protein induction at very low levels cannot be excluded. De Cal et al. (1997b) suggested that *P. oxalicum* partially prevents blocking or collapse of xylem vessels in infected plants. Furthermore, studies pointed to changes in cambium cells and different levels of growth-promoting hormones related to this prevention of blocking. Experiments will be needed to elucidate induced resistance mechanisms different from those studied here.

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Systemic acquired resistance induced by compost and compost water extract in *Arabidopsis*

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Abstract

Arabidopsis thaliana ecotype Columbia was used to investigate mechanisms underlying systemic acquired resistance (SAR) induced by compost. Bacterial speck caused by *Pseudomonas syringae* pv. *maculicola* KD4326 in plants grown in a compost mix was less severe than those in a peat mix. Sprays with compost water extracts reduced disease severity and the pathogen populations in plants grown in a peat mix, but did not further reduce either in plants grown in the compost mix. Activity in compost water extracts was heat stable and passed through a 0.2 µm membrane. Compost did not induce GUS activity driven by a β-1,3-glucanase gene promoter in *Arabidopsis*; but upon pathogen infection the activity was induced to a higher level in plants grown in the compost mix versus those grown in the peat mix. These results indicate that compost-induced SAR involves the potentiation of this defense response rather than its activation.

Introduction

Antagonism, competition and hyperparasitism often are used to describe interactions between microorganisms in the rhizosphere. Recently, induced systemic resistance (Kloepper et al. 1992) has been proposed to describe interactions among the plant host, the pathogen, and bio-inducers. Some of these inducers predominate in edaphic niches of compost-amended substrates suppressive to Pythium root rot. They decline as organic matter decomposes and suppression to Pythium root rot is lost (Boehm et al. 1993). Zhang et al. (1996) showed that systemic acquired resistance (SAR) may play a role in the disease suppression mediated by compost. The objective of this work was to study the mechanisms underlying SAR in plants induced by compost mixes or compost water extracts.

Materials and Methods

Arabidopsis thaliana ecotype Columbia 28-day-old seedlings were grown in a compost or peat mix prepared as described previously (Zhang et al. 1996). Treatments were plants sprayed with (i) distilled water, (ii) 1 mM salicylic acid, (iii) a compost water extract, (iv) a peat water extract, (v) a filter-sterilized compost water extract, and (vi) a filter-sterilized peat extract. Water extracts were prepared by adding tap water to the mixes (1 : 1, v/v) and were incubated at 25 °C for a week. Inoculum of *Pseudomonas syringae* pv. *maculicola* KD4326 (Dong et al. 1991) was produced by growing the bacterium 48 h at 25 °C in 0.1 strength King's B broth (King et al. 1954) containing rifampicin at 50 mg/liter. Seedlings were sprayed

with washed inoculum of *P. syringae* pv. *maculicola* and incubated for an additional 4 days. Severity of bacterial speck was determined 4 days later by measuring the percentage of leaves in each pot affected by necrosis. Populations (CFU/g) of *P. syringae* pv. *maculicola* were determined immediately thereafter.

Transgenic A. thaliana plants containing a chimeric reporter gene (BGL2::GUS) were used to monitor the induction of the β-1,3-glucanase gene expression by measuring GUS activity (Cao et al. 1994). GUS activity in plants was detected histochemically by staining 2 days after the last spray. Leaves of 28-days-old plants were excised, surface sterilized in 1% sodium hypochlorite solution for 15 min, washed and stained at 37 °C overnight, and then dehydrated in 75% ethanol overnight and photographed. A quantitative fluorometric assay for GUS activity was conducted according to the procedure of Jefferson et al. (1987).

All experimental data were subjected to analysis of variance. When a significant ($P = 0.05$) F test was obtained for treatments, separation of means was accomplished using Fisher's least significant different ($LSD_{0.05}$) test.

Table 1. Efficacy of compost water extract and of salicylic acid for control of bacterial speck caused by *Pseudomonas syringae* pv. *maculicola* KD 4326 on *Arabidopsis thaliana* ecotype Columbia grown in a composted pine bark or a peat mix.

Spray treatment	Mix treatment			
	Peat	Compost	Peat	Compost
	Disease severity[w] (% necrotic leaves per pot)		Psm ES4326[x] (10^7 CFU/g fresh leaf)	
Uninoculated control	0	0	ND[y]	ND
Distilled water	74.8 a	14.8 de	4.49 a A	1.18 a B
Compost water extract	28.1 c	19.9 d	0.94 bc A	0.50 a A
Sterilized compost water extract[z]	35.9 b	19.4 d	0.34 c A	0.86 a A
Salicylic acid	19.6 d	10.9 e	2.14 ab A	1.29 a A

[w] Disease severity of bacterial speck (*P. syringae* pv. *maculicola* ES4326) expressed as percentage of necrotic leaves per pot (n = 6). Statistical analysis for disease severity indicated a significant ($P = 0.05$) interaction between spray and mix treatments. Means followed by different letters are significantly ($P = 0.05$) different according to the $LSD_{0.05}$ test.

[x] Means of populations of *P. syringae* pv. *maculicola* ES4326 for mix and spray treatments were separately tested because no significant ($P = 0.05$) interaction occurred between these two treatments. According to the $LSD_{0.05}$ test, means in the same column followed by different lower-case letters are significantly different ($P = 0.05$). Upper-case letters indicate significant ($P = 0.05$) differences between the mix treatments for each spray treatment.

[y] ND = not detected.

[z] Compost water extract was filter-sterilized (0.2 µm). Sterility was confirmed on 0.1 strength Tryptic Soy agar.

Results

Bacterial speck severity and the populations of *P. syringae* pv. *maculicola* (Table 1) in plants grown in the compost mix were lower (P = 0.05) than in those grown in the peat mix. The unfiltered and filter-sterilized compost water extracts decreased disease severity and *P. syringae* pv. *maculicola* populations in plants in the peat mix as compared to those sprayed with water. Salicylic acid did not affect *P. syringae* pv. *maculicola* populations though decreased disease severity in plants grown in the peat mix. In the compost mix, however, none of the spray treatments affected disease severity nor *P. syringae* pv. *maculicola* populations. Activity in the compost water extract was heat-stable and passed through a 0.2 µm membrane.

Plants grown in the compost mix had higher GUS activity than those in the peat mix (Table 2). GUS activity was also higher in plants sprayed with salicylic acid and compost water extracts compared with those sprayed with water or peat water extracts.

Table 2. GUS activity induced by potting mix water extracts or salicylic acid in *Arabidopsis thaliana* ecotype Columbia produced in the composted pine bark or the peat mix.

Spray treatment	Mix treatment[x]		
	Compost	Peat	Mean[y]
	GUS activity (pmol MU min/mg of protein)[z]		
Salicylic acid	53.1	32.5	42.8 a
Compost water extract	48.4	33.3	40.8 a
Sterilized compost water extract	35.8	21.8	28.8 ab
Peat water extract	21.1	22.5	21.8 b
Sterilized peat water extract	21.3	18.8	20.0 b
Distilled H_2O	25.8	12.2	19.0 b
Overall mean[y]	34.2 a	23.5 b	

[x] Spray and potting mix treatments significantly (P = 0.05) affected GUS activity. No significant interaction was observed.

[y] Spray treatment means or mix treatment means followed by different letters are significantly (P = 0.05) different according to the $LSD_{0.05}$ test.

[z] GUS activity determined by a fluorometric assay (TKO 100 Mini Fluorometer, Hoefer Scientific Instruments, San Francisco, CA) according the procedure described by Jefferson et al. (1987).

Discussion

Induction of SAR in cucumbers by composts appears to be biological in nature. In a previous study we observed and demonstrated the loss of activity with autoclaving. The mechanisms by which the compost water extracts used in this work induced disease suppression appear to be different from that of the compost mix used to grow the plants. Suppression of *A. thaliana* bacterial speck was more likely due to SAR induced by the compost mix. It is consistent with our previous studies on the suppression of Pythium root rot and anthracnose of cucumber in this mix (Zhang et al. 1996).

The pattern of GUS gene expression induced by the compost mix differed from that induced by pathogen infection and salicylic acid, and even from that induced by compost water extracts. Most of the increase in GUS activity induced by compost did not occur until after the plant became infected with the pathogen. SAR induced by various agents involves

extensive and continuing exchanges of information evoking complex host response to the inducers. These responses determine the outcome of the interactions among the host plant, the pathogen, biocontrol agents, and environmental conditions. Biocontrol agents in compost may be involved in the potentiation of resistance responses rather than high level activation. The timing and magnitudes of these responses to pathogen attack have great impacts on the outcome of such interactions. Raaijmakers et al. (1995) showed that PGPR-mediated activity is dose-dependent. Thus when, where, and how one measures SAR induced by biocontrol agents in plants may lead to different observations and different conclusions.

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Silicon induces the accumulation of antifungal low-molecular-weight metabolites in cucumber plants

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Abstract

The role of silicon in plant resistance to diseases is controversial. It was long considered to protect plants from infection by polymerizing in epidermal cells, thereby increasing cell resistance to penetration by pathogens. However, several recent studies presented evidence for an active role of the element, which seemed to stimulate the expression of defence reactions. In this work, it is shown that silicon is actively implicated in resistance of cucumber to powdery mildew, a foliar pathogen, by enhancing the production of antifungal low-molecular-weight metabolites related to phenolics.

Introduction

Silicon (Si) is the second most abundant element in the earth's crust and is found in most living organisms, in minute amounts. In plants, silicon positively influences their resistance to pathogenic fungi (Bélanger et al. 1995, Epstein 1994), by limiting fungal penetration with specific accumulation and polymerization of $Si(OH)_4$ in the epidermal cell walls (Kunoh 1990). Recent work, however, contended that silicon may also act by stimulating the natural plant defense mechanisms (Bélanger et al. 1995). We further studied this hypothesis by investigating the possible active role of Si as an inducer of disease resistance in the interaction of cucumber (*Cucumis sativus* L.) and powdery mildew (*Sphaerotheca fuliginea* Schlech.:Fr. Poll). We searched for stimulation of antifungal metabolites accumulation in infected leaves after Si fertilization. Previous microscopic analysis of Si effects on cucumber resistance indicated that cells attacked by either *Pythium ultimum* or *S. fuliginea* accumulated fungitoxic phenolic compounds (Chérif et al. 1992, Menzies et al. 1991) similar to accumulation of phytoalexins (Snyder and Nicholson 1990). In this study we provide conclusive evidence that silicon plays an active role in disease resistance by inducing the production/accumulation of antifungal low-molecular-weight metabolites during pathogenesis in cucumber. Silicon-induced metabolites represent the first evidence of flavonoid phytoalexins in cucumber.

Materials and Methods

Plant material and pathogen inoculation. Cucumber cv. Mustang was grown in a growth chamber and provided nutrient solution containing 0 or 100 ppm Si prepared according to Chérif et al. (1991). Plants of the four-leaf stage were inoculated with *Sphaerotheca fuliginea*. Infected leaves were harvested 1, 2, 3, 4, 6, 8 and 10 days after inoculation. The experiment included four treatments: non-infected Si-minus and Si-plus plants (Si^-PM^-, Si^+PM^-), and

powdery mildew (PM) infected silicon-minus and silicon-plus plants (Si^-PM^+, Si^+PM^+).

Samples were extracted overnight in 80 % MeOH. The MeOH was evaporated and the aqueous phase submitted to acid hydrolysis (2 M HCl, 100 °C, 1 h). Aglycones obtained were extracted with ethyl acetate (EtOAc). The resulting fraction was used for thin layer chromatographic analysis (TLC) and for determining antifungal activity of leaf samples. The equivalent of 1.5 g fresh weight of the EtOAc fraction was spotted on a TLC plate and run in EtOAc:dichloromethane (6:4). The TLC plate was first examined under long wave UV light and subsequently used for antifungal determination. Antifungal activity was directly evaluated on TLC plates, *Cladosporium cucumerinum* was sprayed over the plates as a spore suspension in PDA. Plates were incubated in a humid chamber for 2 days at 25 °C.

Isolation of cucumber antifungal low-molecular weight metabolites. To isolate antifungal metabolites, leaves from Si^+ PM^+ plants (approx. 100 g fresh weight) were pooled and extracted for phenolics as described above. The EtOAc fraction obtained was subjected to several steps of purification using preparative TLC and column chromatography. Fractions obtained after each purification step were analyzed for antifungal activity using the *C. cucumerinum* bioassay to eliminate inactive fractions.

Results and Discussion

Silicon stimulated the accumulation of antifungal compounds in infected leaves of cucumber. TLC analysis of cucumber leaf aglycones liberated after acid hydrolysis underscored the appearance of several distinctive compounds following Si fertilization. Throughout the experiment, the pattern of phenolic compounds of control (Si^- PM^-, Si^+ PM^-) and Si^- PM^+ leaves remained similar. A striking difference appeared in the UV fluorescence profile of infected plants as a result of Si fertilization. This difference was due to the accumulation of distinctive bands between $Rf = 0.4$ and 0.6 specific to Si^+ PM^+ plants. They appeared in leaf samples within 1 day of inoculation and remained visible until 4 days after infection. The UV nature of these bands suggested the presence of flavonoids. By comparison, controls and Si^- PM^+ plants only displayed a greenish fluorescing zone in a similar region of the chromatogram.

When tested for antifungal activity, leaf extracts of controls (Si^- PM^-, Si^+ PM^-) and Si^-PM^+ plants showed no major differences at any sampling time. This corroborated their similar UV profile. In contrast, the accumulation of distinctive bands between 1 and 4 days after inoculation in Si^+ PM^+ plants gave rise to an extensive zone of fungitoxicity. In addition, another unique zone of fungitoxicity appeared at Rf=0.0 between 1 and 4 days in Si^+ PM^+ plants (Fawe et al. 1998).

Thus, these results provide evidence that silicon is actively involved in the resistance response of plants to pathogen attack. In cucumber, Si stimulated the synthesis of antifungal compounds that act like phytoalexins. Induction of fungitoxic by Si may contribute to the enhanced resistance of cucumber to powdery mildew, and would partly explain its reported prophylactic properties (Bélanger et al. 1995).

The finding of these presumed phytoalexins prompted us to isolate and identify them. Until very recently (Daayf et al. 1997), Cucurbitaceae were not considered to synthesize phytoalexins as a means of defense.

Cucumber reacted to pathogen attack by producing phytoalexins. Only recently have phytoalexins been identified in Cucurbitaceae and they have not previously been implicated as a defense response. The nature of the Si induced metabolites in Si^+ PM^+ leaf extracts was

examined. Two compounds could be isolated in sufficient quantity for comparison with total extracts and characterization. Based on UV characteristics, both belong to the phenolic class of flavonoids, probably of the flavone type (Fig. 1).

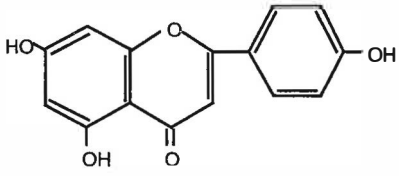

Fig. 1. Chemical structure of the flavone-type flavonoid, apigenin. The UV absorption of flavonoids is attribuable to the aromatic rings and on the hydroxylation pattern. The two compounds isolated in this work appear as deep purple bands under long wave (366 nm) UV light.

In order to assess the biological significance of the compounds, their R*f*, UV behavior and antifungal activity were compared to total leaf extracts. When plated individually, the purified compounds had R*f* values corresponding to the first of the two bands with strong antifungal activity particularly well defined in total leaf extracts of the 4 days Si^+ PM^+ treatment. It had the same UV characteristics and strong fungitoxicity.

Flavonoids are commonly found constitutively in plant tissues. Isoflavonoids and their derivatives represent one of the most studied groups of phytoalexins in the plant kingdom, characterizing the Leguminoseae (Bailey and Mansfield 1982). Despite extensive analysis, phytoalexins were only recently detected in Cucurbitaceae (Daayf et al. 1997). Para-coumaric methyl ester was identified as an antifungal compound constitutively present in healthy tissues, and which increased following infection. We detected flavonoid-type antifungal metabolites in infected cucumber, which behaved like true phytoalexins and were absent from healthy tissues. Clearly there is a need to reconsider the synthesis of phytoalexins as a defense reaction in Cucurbitaceae, possibly in gene-for-gene or systemic acquired resistances.

By demonstrating that silicon amplified the chemical defense of cucumber, this work also raises the challenge to understand the physiology of silicon in plant resistance and also to reexamine its role in monocots, where its known prophylactic properties are still attributed to a passive effect.

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Influence of introduced salicylic acid biosynthesis genes on the disease suppressive capacity of different *Pseudomonas fluorescens* strains

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Abstract

Pseudomonas aeruginosa synthesizes salicylate from chorismate in two reactions catalyzed by isochorismate synthase (the *pchA* gene product) and isochorismate-pyruvate lyase (the *pchB* gene product). The *pchBA* genes are part of the *pchDCBA* operon, which is involved in the biosynthesis of pyochelin and regulated by Fur-mediated iron repression. To study the effects of the plant defense signal salicylate in the rhizosphere, we subcloned the *pchBA* genes into two *Pseudomonas* expression vectors and used these constructs to obtain high level, iron-independent production of salicylate in root-colonizing strains of *Pseudomonas fluorescens*. A *pchBA*$^+$ transgenic derivative of the salicylate-negative strain P3 showed significantly improved capacity to induce systemic resistance in tobacco against Tobacco necrosis virus (TNV). Introduction of the *pchBA* genes into CHA0, a good biocontrol strain, enhanced its salicylic production in the rhizosphere, but did not further enhance its ability to induce resistance against TNV. Both *pchBA*$^+$ transgenic strains P3 and CHA0 showed only marginally improved ability to suppress black root rot of tobacco. In conclusion, salicylate may be one of several bacterial metabolites which together determine the plant beneficial effects of some root-colonizing *Pseudomonas* spp.

Environmental signals

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Induced promoter activity in *Pseudomonas fluorescens* RIWE8 near wheat roots

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Abstract

Colonization and reporter gene expression of an inoculant Tn*5*-B20 (*lacZ* as reporter gene) mutant of *Pseudomonas fluorescens* R2f Rp^r (RIWE8) was studied in soil planted with wheat, under a controlled light and temperature regime. Strain RIWE8 as well as its parent strain R2f Rp^r colonized the wheat rhizosphere and rhizoplane within 2 days after introduction into soil. Reporter gene expression in RIWE8 cells in the wheat rhizosphere was measured from day 7 and it was therefore concluded that the promoter which controls reporter gene expression in RIWE8 responded to root exudates released by the wheat plants in soil. However, RIWE8-CFU counts appeared to be reduced, as compared with corresponding R2f Rp^r counts in rhizosphere soil and on the rhizoplane after 3 weeks, which indicates that survival of the mutant near the wheat roots may be affected by the insertion of the Tn*5*-B20 construct or by reporter gene expression.

Introduction

Roots release substantial amounts of soluble organic materials, denoted as exudates. These exudates are considered to be substrate for many different soil microorganisms, including root pathogens and potential antagonists of these pathogens. Little is known about the specific quality of the release of exudates into soil and the response of saprophytic and pathogenic soil microorganisms.

We constructed a Tn*5*-B20 mutant of *Pseudomonas fluorescens* R2f Rp^r, designated as RIWE8 (van Overbeek and van Elsas 1995), which responded specifically to root exudates of monocotyledons, particularly proline, under in vitro conditions as well as in natural soil. It had been demonstrated that the promoter in RIWE8 was specifically induced near wheat roots under natural (non-sterile) soil conditions in a plant/soil microcosm, where the plant roots were separated from the inoculated soil by nylon gauze (van Overbeek and van Elsas 1995). However, the microcosm set-up used in that study may not be representative for rhizosphere soil under field conditions.

An important difference with conditions in the field and in the microcosm system is that the latter does not enable assessment of differences in root exudate release during plant development. Exudates are not uniformly released along the root axis of growing plants and during the different stages of plant root growth (Curl and Truelove 1986, Gregory and Atwell 1991, Keith et al. 1986). Similarly, this can influence the behavior of pathogens which is often determined by the release of specific compounds from roots (Deacon 1991). Differences in reporter gene activity of RIWE8 can thus be expected during the growth of roots. Information about the timing and extent of induction of the root exudate-induced promoter in RIWE8 near developing roots is, therefore, essential to decide whether the regulatory region identified is

useful as a controlling element of beneficial genes like *cry*IVB (van Elsas et al. 1991, Waalwijk et al. 1991), phenazine-1-carboxylic acid and 2,4 diacetylphloroglucinol (Mazzola et al. 1992, Mazzola et al. 1995, O'Sullivan and O'Gara 1992), which might be placed under the control of exudate dependent promoter region.

Therefore, an experiment was performed with *P. fluorescens* RIWE8 introduced into soil planted with wheat under greenhouse conditions, as a transitional stage from the aforementioned microcosms to the release of RIWE8 in a field plot planted to wheat. The aims of the experiment were to determine whether reporter gene activity can be detected in rhizosphere soil of wheat plants and whether differences in reporter gene activity could be detected during plant development.

Materials and Methods

Culturing of the strains and introduction into soil. *P. fluorescens* strains R2f Rp^r and RIWE8 (van Overbeek and van Elsas 1995) were grown overnight in M9 medium (Na_2HPO_4 x H_2O, 6g; KH_2PO_4, 3g; NH_4Cl, 1g; NaCl, 0.5g; $MgSO_4$ x 7 H_2O, 0.25g; $CaCl_2$ x H_2O, 0.0147g; Millipore membrane filtered H_2O, 1 liter, pH 7) amended with 0.1% glucose at 27 °C.

Cells were harvested by centrifugation (10,000 x *g*, 10 min, room temperature), washed (three times) in sterile Millipore membrane filtered water and suspensions of the washed cells were subsequently introduced into air-dried (moisture content 10%) and sieved (4-mm mesh) Flevo silt loam (FSL) soil, establishing a soil moisture content of 28%. Shortly (3 h) after introduction into soil, the R2f Rp^r and RIWE8 CFU numbers were at 8.13 and 8.07 per g of dry soil respectively.

Plant/soil microcosm experiments and soil sampling. Polypropylene beakers were filled with 100 g moist soil inoculated with R2f Rp^r or RIWE8, planted with three 3-day-old wheat (*Triticum aestivum* cv. Sicco) seedlings and incubated in the greenhouse at 20 °C with 24 h light. Every two days, soil moisture was gravimetrically corrected to the original moisture content (28%) by the addition of sterile demineralized water.

At 2, 7 and 21 days after planting of seedlings, triplicate microcosms containing either R2f Rp^r or RIWE8, were used for CFU enumeration and determination of reporter gene activity as described below. For that purpose, roots were separated from non-rhizosphere (bulk) soil and clumps of soil loosely adhering to the roots were removed. Roots with firmly adhering soil and root-free soil were regarded as the rhizosphere and bulk soil, respectively.

CFU recovery and β-galactosidase activity determination. Samples of 1 g of root with firmly adhering soil as well as 1 g bulk soil samples were aseptically transferred to tubes containing 9 ml of gelatin-phosphate solution [gelatin, 1 g; $(NH_4)_2$ HPO_4, 12 g; demineralized water, 1 liter, pH 10.3; autoclaved for 10 min at 121 °C] and 1 g gravel for flocculation. The tubes were vortexed (1 min) and left to flocculate soil particles (10 min). Samples of the supernatant were taken for CFU counts and β-galactosidase activity determinations.

Roots were subsequently removed from the soil suspensions, rinsed in sterile demineralized water to remove any adhering soil, placed in 9 ml 0.1% sodium pyrophosphate (PP_i)solution and macerated by grinding in sterile 15 ml Potter tubes. Samples from macerated roots were regarded as being representative for the rhizoplane.

Soil or macerated root suspensions (in gelatin-phosphate and 0.1% sodium PP_i solution respectively) were diluted in 0.1% sodium PP_i, after which aliquots of the dilutions were plated onto M9 agar (M9 supplemented with 1.2% technical agar) containing 50 µg/ml

rifampicin and 100 µg/ml cycloheximide (selective for R2f Rp^r) or 50 µg/ml rifampicin, 100 µg/ml cycloheximide and 50 µg/ml kanamycin (selective for RIWE8). Plates were incubated for 2 days at 27 °C prior to colony enumerations. Counts were expressed as CFU/g of soil dry weight (bulk and rhizosphere soils) or as CFU/g fresh root weight (rhizoplane).

Aliquots (1 ml) of cleared soil suspensions in gelatin-phosphate solution were centrifuged (12,000 x *g*, room temperature) and the pellets frozen at -20 °C for β-galactosidase activity determinations. β-Galactosidase activity in thawed pellets was determined by chemiluminescence using the Galacto-Light Plus^{TM} detection method [Tropix, Bedford, MA], according to the manufacturer's instructions (van Overbeek et al. 1997), as well as by enzymatic hydrolysis of o-Nitrophenyl β-D-galactopyranoside (ONPG) (Miller 1972, van Overbeek and van Elsas 1995). Relative light units (RLU) were integrated during 45 s with a Bio-Orbit 1253 luminometer connected to a personal computer using the LUMICOM communication programme [version 1.0, Bio-Orbit, Finland].

The β-galactosidase activity in each soil sample was expressed in light emission units (LEU), determined as the RLU, divided by the log CFU number present in 1 ml cleared soil suspension.

β-galactosidase activity in the cell suspensions of R2f Rp^r and RIWE8, shortly before inoculation into the soil, was at background level (2 and 4 Miller units [MU] respectively), indicating the absence of gene expression in the inoculant cells before introduction into soil.

Statistical analyses. Statistical analyses were performed on triplicate samples. Comparisons were made by analysis of variance (Genstat 5; Rothamsted Experimental Station, Harpenden, United Kingdom) and standard errors of difference were calculated. Values were considered to be significantly different at a 95% (or higher) confidence level. CFU numbers (rhizoplane, rhizosphere and bulk soils) and β-galactosidase activities (rhizosphere and bulk soils) were taken into consideration as experimental factors during the course of the experiment.

Results and Discussion

Pseudomonas fluorescens R2f Rp^r and RIWE8 CFU numbers decreased in bulk soil from log 8.06 and log 7.95 CFU/g of dry soil, 2 days after introduction, to log 5.44 and 5.35 CFU/g of dry soil, three weeks after introduction respectively (Table 1). Also in rhizosphere soil, the CFU numbers of both strains dropped, although after three weeks the CFU counts of both strains in rhizosphere soil were significantly higher than in bulk soil. Differences in CFU counts between strains were not observed in bulk or in rhizosphere soil except on day 21, when numbers of R2f Rp^r were significantly higher than those of RIWE8 in the rhizosphere. CFU in rhizoplane samples progressively decreased from day 2 to day 21 for both R2f Rp^r and RIWE8. Differences in numbers between strains were not observed after 2 and 7 days, however R2f Rp^r CFU numbers at day 21 were significantly higher than those of RIWE8 on the rhizoplane.

Survival of mutant RIWE8 in bulk soil was not affected by the insertion of Tn*5*-B20, as previously observed (van Overbeek and van Elsas 1995). However, this seems to be in contrast to the aforementioned colonization of the rhizosphere and rhizoplane by the bacteria where the numbers of RIWE8 were lower on day 21 than those of the parent strain R2f Rp^r. The reduced colonization may be caused by insertional inactivation of genes which are essential for long-term establishment of the bacteria in the rhizosphere. Possibly, the genes controlled by the proline responsive promoter of RIWE8 are necessary for the uptake and

metabolism of proline from root exudate. However it cannot be ruled out that the induced synthesis of β-galactosidase or the constitutive expression of the marker gene *nptII* cause an extra metabolic load for the RIWE8 cells in sites near and on the root.

Table 1. Survival and β-galactosidase activity of *P. fluorescens* R2f Rp^r and RIWE8 in soil microcosm planted to wheat.

Days	R2f Rp^r					RIWE8				
	log CFU count (log CFU/g of dry soil)		activity (LEU)[z]		log CFU count (log CFU/g of root)	log CFU count (log CFU/g of dry soil)		activity (LEU)		log CFU count (log CFU/g of root)
	bulk	rhizo-sphere	bulk	rhizo-sphere	rhizoplane	bulk	rhizo-sphere	bulk	rhizo-sphere	rhizoplane
2	8.06	8.18	0.22	0.60	6.51	7.92	7.95	0.45	0.59	6.49
7	7.53	7.44	0.30	0.66	5.63	7.43	7.35	0.43	1.07	5.56
21	5.44	6.89	0.68	1.53	6.08	5.35	6.32	0.62	2.34	5.32

[z] LEU: Light emission units, i.e. relative light emission units as determined by chemiluminescence (Galacto-Light Plus™, Tropix) divided by the log CFU number present in the sample. Least significant difference CFU count bulk and rhizosphere, 0.50; Least significant difference activity; 0.22; Least significant difference CFU count rhizoplane: 0.28.

Induction of the proline responsive promoter and reporter gene expression in RIWE8 in the rhizosphere could be observed by increased hydrolysis of ONPG and the chemoluminometric approach (Table 1). β-galactosidase activities in R2f Rp^r inoculated rhizosphere soil, determined by ONPG hydrolysis, were high (between 14 and 58 MU') in comparison with that in corresponding bulk soil (between 3 and 10 MU'). These measured activities were not different from RIWE8 inoculated rhizosphere (between 19 and 69 MU') and bulk (between 4 and 13 MU') soils. Determination of β-galactosidase activities in R2f Rp^r inoculated soil samples using chemoluminometric detection did not reveal this background. The high activity measured in R2f Rp^r inoculated rhizosphere soil by the ONPG method may be due to β-galactosidase-independent hydrolysis of ONPG from wounded roots. In another study, β-galactosidase activity measurements by the chemoluminometric approach also appeared to be more sensitive than by ONPG hydrolysis (van Overbeek et al. 1997, van Poucke and Nelis 1995). It was therefore concluded that more accurate measurements of reporter gene expression in RIWE8 cells near growing roots in soil are to be obtained by chemoluminometric detection of β-galactosidase.

β-galactosidase activities in bulk soil between both strains was not significantly different when measured by the chemiluminescent method. However in rhizosphere soil, induction of reporter gene activity in RIWE8 was higher than that in R2f Rp^r from day 7 (Table 1). An increase in activity was observed in R2f Rp^r inoculated rhizosphere soil ranging from 0.6 LEU (day 2) to 1.53 LEU (day 21) and in RIWE8 inoculated rhizosphere soil from 0.59 LEU (day 2) to 2.34 LEU (day 21). The increase of β-galactosidase activity in R2f Rp^r inoculated rhizosphere soil also has been observed previously (van Overbeek and van Elsas 1995). It can be explained by an increase of the numbers and/or activity of *lacZ* containing indigenous

bacteria in rhizosphere soil as a result of the introduction of R2f Rp^r cells into soil. Namely, a part of the introduced population will die and lyse and the released cellular components will become available as nutrients for indigenous soil species.

From these experiments we conclude that reporter gene expression in RIWE8 can be observed in a realistic and complex environment on growing roots, extending the observations made in the model plant/soil microcosm set-up (van Overbeek and van Elsas 1995) to a more realistic system. Differences in colonization between R2f Rp^r and RIWE8 became apparent at day 21 suggesting that colonization of mutant RIWE8 was affected during the development of the seedlings.

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A Fusarium pathogenicity factor blocks antibiotic biosynthesis by antagonistic pseudomonads

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Abstract

Fusaric acid, a non-specific pyridine-carboxylic acid phytotoxin produced by *Fusarium oxysporum*, was identified as a negative signal in biocontrol of Fusarium crown and root rot of tomato. Fusaric acid (100 µg/ml) repressed production of the antibiotic 2,4-diacetylphloroglucinol (PHL) by some but not all *Pseudomonas fluorescens* biocontrol strains. Strains sensitive to fusaric acid repression had significantly lower biocontrol activity. Production of PHL was a primary mechanism of action for strain Q2-87 which was not sensitive to fusaric acid; whereas, PHL had no role in CHA0 which is sensitive to fusaric acid. The residual level of control with this strain was attributable in part to production of the biocide hydrogen cyanide (HCN).

Introduction

Fusarium oxysporum f.sp. *radicis-lycopersici* causes crown and root rot of tomato, an increasingly important disease in hydroponics production systems. Fluorescent pseudomonads have been effective for biocontrol of this and other *Fusarium* diseases. However, certain biocontrol strains tend to perform better than others. Our objective was to determine the molecular basis behind this variation with the long-term aim of improving strain selection procedures.

Materials and Methods

We screened an ecologically and genetically diverse collection of 42 *Pseudomonas fluorescens* strains for biocontrol of Fusarium crown and root rot of tomato. All bacteria carry a conserved *phlD* gene essential for biosynthesis of the antibiotic PHL. In addition, all strains produce HCN. Keel et al. (1996) characterized these strains into three distinct groups based on amplified ribosomal DNA restriction analysis (ARDRA).

In this study, we compared the strains for biocontrol activity against crown and root rot in a soilless rockwool system as described by (Duffy and Défago 1997). Tomato seeds (*Lycopersicum esculentum* cv Bonnie Best) were placed in rockwool blocks in shallow trays, and the rockwool was saturated with a mineral nutrient solution. The nutrient solution was inoculated with both the pathogen (10^6 microconidia plus mycelial fragements per ml) and the bacterium (10^7 CFU per ml). After 2 weeks growth in the greenhouse, plants were removed from the rockwool and disease was evaluated using a scale of increasing severity from 0 to 4.

Then, we determined the influence of fusaric acid, a pathogen phytotoxin, on bacterial antibiotic production. Bacteria were grown in liquid PCG medium (Duffy and Défago 1997) with and without 100 µg/ml fusaric acid (Sigma Chemical Co., St. Louis, MO, USA). PHL

production and fusaric acid degradation was quantified after 48 hours using HPLC as previously described (Duffy and Défago 1997). The minimum-concentration of fusaric acid needed to repress PHL production was determined with strain CHA0. CHA0 was grown 48 h in PCG medium amended with fusaric acid at 0-200 µg/ml and PHL production was quantified as above. The relationship between biocontrol activity and sensitivity to fusaric acid was evaluated using SAS regression procedures (SAS Institute, Cary, NC, USA). Disease severity rating was plotted against the amount of PHL produced in the presence of fusaric acid.

Finally, the relative role of PHL and HCN in biocontrol was determined with metabolite-negative insertion mutants and restored mutants of CHA0 and Q2-87 (Vincent et al. 1991, Voisard et al. 1989). Bacteria were compared with the wild-type strains in the rockwool assay as described above. Pathogen sensitivity to PHL was determined after 7 days growth on 2% malt extract agar amended with 0-150 µg/ml synthetic antibiotic following Keel et al. (1992).

Results and Discussion

An ecologically and genetically diverse collection of 42 *Pseudomonas fluorescens* biocontrol strains was characterized into three groups using ARDRA analysis (Keel et al. 1996). There was a wide variation between strains in the level of biocontrol provided against Fusarium crown and root rot of tomato in a rockwool soilless system. This variation was attributed to differential responses of the strains to fusaric acid, a pathogen phytotoxin. Fusaric acid repressed production of PHL by all ARDRA 1 strains but did not affect bacterial growth. For example, antibiotic production by strain CHA0 was completely repressed with 0.12 µg/ml fusaric acid (Fig 1) and concentrations as high as 200 µg/ml did not reduce total CFU (data not shown). Strains in ARDRA 2 and 3 groups were less sensitive or resistant to fusaric acid repression. Regression analysis demonstrated a significant inverse relationship between sensitivity to fusaric acid in vitro and biocontrol activity (data not shown). In other words, sensitive strains were inferior whereas resistant strains were superior biocontrol agents.

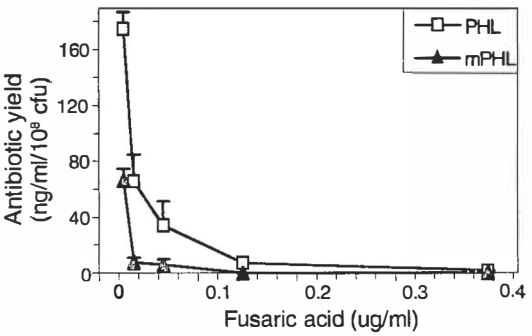

Fig. 1. Fusaric acid repression of 2,4-diacetylphloroglucinol (PHL) and the precursor antibiotic mono-acetylphloroglucinol (mPHL) in *P. fluorescens* CHA0 after 48 h in PCG liquid medium amended with fusaric acid (0-200 µg/ml). At concentrations above 0.37 µg/ml, no antibiotics were detected using HPLC. (From Duffy and Défago 1997)

Fusarium oxysporum was sensitive to pure PHL (ID_{50} between 30 and 50 µg/ml). This suggests that PHL production could confer a competitive advantage to pseudomonads over the pathogen. Indeed, PHL production is a primary mechanism of biocontrol in fusaric-acid resistant strains. Genetic interuption of PHL biosynthesis genes in Q2-87 substantially reduced biocontrol activity (Table 1). In contrast, PHL had little if any role in biocontrol in

the fusaric-acid sensitive strain CHA0. In this strain, HCN production contributed to the moderate level of biocontrol observed. This highlights the advantage of having multiple biocontrol mechanisms on hand to cope with different environmental conditions.

Table 1. Relative role of 2,4-diacetylphloroglucinol (PHL) and hydrogen cyanide (HCN) in biocontrol of Fusarium crown and root rot by *Pseudomonas fluorescens*

Strain	Phenotype	Disease rating (0-4)	Plant fresh weight (mg)
None	------	2.80 (0.16)	130.6 (2.8)
CHA0 wt	PHL+, HCN+	2.41 (0.10)	170.2 (12.6)
CHA630	PHL minus, HCN+	2.53 (0.05)	160.2 (11.1)
CHA630/pMON5118	PHL restored, HCN+	2.33 (0.07)	165.3 (7.3)
CHA5	PHL+, HCN minus	2.65 (0.13)	142.2 (11.4)
CHA5/pME3013	PHL+, HCN restored	2.42 (0.06)	175.4 (13.0)
Q2-87 wt	PHL+HCN+	1.41 (0.11)	239.2 (7.8)
Q2-87::Tn5-1	PHL minus, HCN+	2.48 (0.11)	162.6 (13.0)
Q2-87::Tn5-1/pMON5118	PHL restored, HCN+	1.52 (0.10)	218.3 (6.7)

Values (± SE) represent the means of 5-6 replications with 12 plants each. All treatments were challenged with *F. oxysporum* f.sp *radicis-lycopersici* and evaluated after 2 weeks.

Our findings have practical applications for strain selection. Raaijmakers et al. (this volume) recently outlined a screening procedure for biocontrol pseudomonads based on selection of PHL-producing strains using a *phlD* molecular probe. We suggest that the screening can be further refined by selecting strains capable of expressing these genes in particular environments. In the case of strain selection for biocontrol of *Fusarium* diseases, it would be important to select not only for PHL producing strains but for PHL strains with the capacity to produce this antibiotic in the presence of the pathogen. We further observed that biocontrol was not related to the amount of PHL produced in the absence of fusaric acid. This strengthens the argument that screening based on in vitro production of antimicrobial compounds should be evaluated qualitatively, rather than quantitatively. Strains that produce low levels in vitro in a particular medium, may produce much more under other conditions more relevant to biocontrol. Indeed, the quantity of PHL produced by the strains used in our study differed greatly when evaluated in different media (PCG liquid in this study, KMB and malt extract agar in Keel et al. 1996).

This study also shows that even though biosynthetic genes may be conserved among strains, the regulation of these genes can differ dramatically. Continuing efforts to identify these differences will shed new light on antibiotic regulation. Ultimately, this may lead to novel approaches for improving bacterial interactions with the environmental conditions under which they must operate.

Acknowledgments

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Oregon, USA; F. O'Gara, Cork University, Ireland; L. Thomashow and D. Weller, USDA, Pullman, Washington, USA for providing bacterial strains; and U. Berger for providing antibiotic standards for quantitative HPLC.

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Zinc amendments increase antibiotic biosynthesis and improve genetic stability in biocontrol pseudomonads

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Abstract

Abiotic soil factors such as zinc influence the reliable performance of bacterial biocontrol agents (Phytopathology 80:995). Increasing the availability of favorable factors is one approach to improve biocontrol. Zinc amendments have previously been shown to enhance suppression of tomato and wheat root diseases by Pseudomonas fluorescens (Phytopathology 86:S36). We have now found that zinc stimulated production of 2,4-diacetylphloroglucinol antibiotic by some biocontrol pseudomonads but not others while glucose increased production by all strains. Zinc improved the stability in these bacteria of regulatory genes for antibiotic biosynthesis (*gacA* and *lemA*). Biocontrol strains were differentially tolerant to zinc concentrations highlighting the need for judicial use of mineral amendments to avoid nontarget effects on other beneficial microbes. The influence of zinc on production of antibiotics which contribute to the ecological competence of pseudomonads, further indicates that this trace mineral is a key environmental signal in biocontrol.

Signal transduction and the two-component regulatory system encoded by the *LemA/GacA* genes in *Pseudomonas fluorescens* CHA0: implications for biological control

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Abstract

Pseudomonas fluorescens CHA0 produces several secondary metabolites, many of which have been implicated in the biological control of fungal pathogens of important crop plants. The production of extracellular protease and secondary metabolites including hydrogen cyanide and a number of antibiotics has been shown to be regulated by a complex mechanism involving a two-component regulatory system known as LemA/GacA. The global regulator GacA is essential for antibiotic and exoenzyme production and for biocontrol activity in *P. fluorescens*. The other component of this regulatory system, the cognate sensor, is closely related to LemA, a protein which controls toxin and protease expression in *P. syringae*. The *lemA* gene is necessary for the control of secondary metabolism in CHA0 and is likely to be essential for biocontrol activity. Tn*5*-induced *lemA* mutants of CHA0 that fail to produce secondary metabolites are fully restored to wild-type activity when complemented by the *lemA* gene of *P. syringae*. Regulatory proteins that control the production of compounds essential for pathogen inhibition are obvious targets for genetic manipulation of biocontrol strains. In this study, a primary objective is to understand at the molecular level the mechanism of signal transduction by the *lemA/gacA* system.

We report the identification and characterisation of the *lemA* gene fromCHA0. Several CHA0 mutants were constructed whereby the wild-type *lemA* gene was replaced by derivatives containing domain-specific mutations within *lemA*, recombined into the CHA0 chromosome. The effects of these specific mutations on exoproducts was then analysed using, in addition constructs containing *lemAgacA* regulated promoters fused to a *lacZ* reporter.

Abiotic stresses induce non-culturability in the biocontrol strain of soil-borne diseases, *Pseudomonas fluorescens* CHA0

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Abstract

The bacterium *Pseudomonas fluorescens* strain CHA0 produces several antimicrobial compounds and protects plants from different soil-borne diseases. The ability of biocontrol bacteria to colonize and to persist in the soil is recognized as a key factor for efficient biocontrol. Abiotic stresses are believed to be among the major factors affecting survival and persistence of the inoculant in the soil. In the present study, we incubated a spontaneous rifampicin-resistant mutant of the strain (CHA0-Rif) under different stress conditions *in vitro* and monitored its survival.

Cells were exposed to high salt concentrations (0.7 and 1.5 M NaCl), to low redox potential achieved with potassium hexacyanoferrate (Ep = 230 mV), and anaerobic conditions, as well as acidic conditions (pH 3 and pH 4) for one week. Determinations included total cell counts by immunofluorescence microscopy, viable counts using Kogure's substrate responsiveness test, and colony counts on rifampicin containing plates.

Results indicated that harsh abiotic stresses like 1.5 M NaCl, anaerobic conditions in combination with a low redox potential and acidic pH induced non-culturability in CHA0-RIF cells. However, numbers of total cells were hardly affected by the stress and large number of the stressed cells remained even viable.

Overall, stress conditions induce change in the physiological status of *Pseudomonas fluorescens* CHA0. Further experiments are required to study which exogenous stimuli are able to resuscitate non-culturable cells in order to evaluate their biocontrol activity and environmental impact.

Population structure and ecology

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Plant protection by antibiotic-producing bacteria in natural disease-suppressive soils

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Abstract

Evidence has been accumulating for the last two decades that antibiotic-producing fluorescent *Pseudomonas* spp. may play a key role in soils that are naturally suppressive to certain soil-borne plant pathogens. Our studies have focused on the role of phenazine (Phz) and 2,4-diacetylphloroglucinol (PHL) producing *Pseudomonas* spp. in soils that are naturally suppressive to take-all of wheat. Through the use of primers and probes directed against specific sequences within the biosynthetic loci for Phz and PHL, we have demonstrated that PHL-producing fluorescent *Pseudomonas* spp. are highly enriched on roots of wheat grown in at least five different take-all suppressive soils but not on roots grown in the corresponding take-all conducive soils. Phenazine-producing fluorescent *Pseudomonas* spp. were not detected on roots of wheat grown in any of the soils tested. We suggest that PHL producers play a key role in the natural biological control that operates in take-all suppressive soils in Washington State, USA. PHL-producing fluorescent *Pseudomonas* spp. also have been readily isolated from soils suppressive to black root rot of tobacco and from soils suppressive to Fusarium wilt of tomato, suggesting that these antibiotic-producing bacteria also may play an important role in the natural plant defense that operates in other disease-suppressive soils.

Introduction

Interest in biological control of plant pathogens has been stimulated in recent years by trends in agriculture towards greater sustainability and public concern about the use of hazardous chemical pesticides. Research strategies which have combined molecular biology approaches with more traditional microbiological methods have significantly advanced our understanding of the mechanisms involved in suppression of plant pathogens by beneficial microorganisms. There is now unequivocal evidence that metabolites including antibiotics, siderophores, and volatiles produced in situ by plant growth-promoting rhizobacteria (PGPR) play key roles in the control of various soil-borne plant pathogens (O'Sullivan and O'Gara 1992, Thomashow and Weller 1995). PGPR also may benefit the host plant directly through the production of metabolites that either stimulate root development and plant growth (Glick 1995) or trigger the induction of systemic acquired resistance (De Meyer and Hofte 1997, Hoffland et al. 1995, Liu et al. 1995, Pieterse et al. 1996, Press et al. 1997, Van Wees et al. 1997, Wei et al. 1996).

A breakthrough in biological control of soilborne plant pathogens appears to have resulted from renewed attention to the natural suppressiveness of certain soils to plant pathogens (Cook and Baker 1983). Many genera of bacteria and fungi have been implicated in the natural suppressiveness of soils to pathogens. Among the fungi, *Trichoderma* spp. and

nonpathogenic *Fusarium* spp. appear to contribute to suppressiveness of soils to *Rhizoctonia solani* (Chet and Baker 1981, Papavizas 1985) and to pathogenic *F. oxysporum* (Alabouvette 1990, Larkin et al. 1996), respectively. Among the rhizobacteria, the fluorescent *Pseudomonas* spp. have been implicated in the suppressiveness of soils to *F. oxysporum* (Lemanceau and Alabouvette 1993) and to take-all of wheat (Cook and Rovira 1976, Raaijmakers et al. 1997, Weller 1988). In this paper, we will focus on the role of antibiotic-producing fluorescent *Pseudomonas* spp. in natural disease-suppressive soils and in particular on their role in take-all suppressive soils.

Natural disease-suppressive soils

Most natural soils are suppressive to some degree to soilborne plant pathogens. This widespread characteristic has been referred to as "general suppression" (Gerlagh 1968) or "general antagonism" (Hornby 1983). When a given amount of inoculum of a pathogen is added to raw or sterilized soil, the effect of general suppression is apparent by the greater severity of disease on a host grown in sterilized as compared to raw soil. General suppression appears to be related to the total amount of microbial activity and biomass in soil and often is increased by the addition of organic matter, certain cultivation practices, and fertilizers. Disease-suppressive soils were defined by Baker and Cook (1974) as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil". The 'specific suppression' that operates in natural disease-suppressive soils is superimposed over the background of general suppression and is due at least in part to the specific effects of individual or select groups of microorganisms that adversely affect the pathogen during some phase of its life cycle.

Suppressive soils have been described for a variety of plant pathogens and parasites, including *Gaeumannomyces graminis* var. *tritici*, *F. oxysporum*, cereal cyst nematode (*Heterodera avenae*), *Phytophthora cinnamomi*, *Pythium* spp., *R. solani* and *Streptomyces scabies* (Cook and Baker 1983, Hornby 1983, Huber and Schneider 1982, Roget 1995). Although most studies have focused on soils with microbiologically based suppression, suppressiveness due strictly to physical and chemical factors also occurs (Meyer and Shew 1991). Hornby (1983) divided suppressive soils into the categories of 'long standing suppression' or 'induced suppression'. Long standing suppressiveness is a condition naturally associated with the soil. Its origin is not known and it appears to survive in the absence of plants. Induced suppressiveness is initiated and sustained by crop monoculture or the addition of inoculum of the target pathogen. Soils suppressive to the formae speciales of *F. oxysporum* which cause wilt diseases of a wide variety of crops (Alabouvette 1990) are the best examples of long standing suppressiveness. Fusarium wilt suppressive soils are known throughout the world. The specific suppressiveness has been related to the activity of fluorescent *Pseudomonas* spp. and nonpathogenic *Fusarium* spp. Competition for nutrients, particularly involving iron chelation, has been proposed as a mechanism in disease suppression by fluorescent pseudomonads (Lemanceau and Alabouvette 1993). Mechanisms of suppression proposed for nonpathogenic fusaria include competition for nutrients and infection courts and induced systemic resistance (Larkin et al. 1996, Lemanceau and Alabouvette 1993, Mandeel and Baker 1991).

Take-all suppressive soils

Take-all, caused by the fungus *G. graminis* var. *tritici*, is one of the most important root diseases of wheat worldwide. Although wheat is particularly susceptible to the take-all fungus, various other Gramineae like barley, rye and triticale can also be infected (Gutteridge et al. 1993). Traditionally, take-all has been controlled by a combination of crop rotation and tillage, practices which reduce the inoculum potential of the pathogen. However, because long rotations are often not economically feasible, and tillage contributes to soil erosion, the current trend in cereal production is toward less tillage and two or three consecutive wheat crops before a break. Both of these practices exacerbate take-all. Breeding wheat for resistance to the take-all fungus has been unsuccessful, and also methods of chemical control are limited.

Take-all decline (TAD) is a natural biological control of take-all and is defined as a spontaneous reduction in disease and increase in yield with extended monoculture of wheat or barley (Slope and Cox 1964). Soils in the state of TAD are suppressive to the disease. TAD was first observed more than 50 years ago (Fellows and Ficke 1934, Glynne 1935) and is now recognized as a worldwide phenomenon (Hornby 1983). The similarity of TAD throughout the world is remarkable in view of the broad range of soil types, climates and agronomic conditions under which wheat is cultivated. Field studies have clearly indicated that the development of TAD follows a consistent pattern everywhere, apparently requiring the continuous cultivation of wheat and the presence of the take-all fungus. Factors such as soil type and previous cropping history seem only to modulate the extent and speed of development of TAD (Shipton 1975). Despite the fact that the incidence and severity of take-all eventually declines, most growers abandon monoculture prematurely because interim losses can be considerable. Once established, however, TAD permits a recovery in yield of wheat and it persists as long as monoculture continues.

The specific suppression that operates in TAD soils: (i) develops with wheat monoculture following one or more outbreaks of take-all; (ii) may develop with the addition of mycelium of the pathogen to soil; (iii) is eliminated by treatment of the TAD soil with moist heat (60 °C, 30 min), methyl bromide or chloropicrin; and (iv) is transferable to conducive soils (1 to 10% suppressive soil added to conducive soil transfers suppressiveness) (Cook and Rovira 1976, Cook and Weller 1987, Shipton et al. 1973). Once TAD develops, the suppressiveness of the soil can be eliminated by breaking the cycle of wheat or barley with an alternate crop (Cook 1981). However, fields with a long history of monoculture quickly regain suppressiveness following an alternate crop once wheat is again grown. Understanding the fundamental mechanism(s) of TAD can be exploited to develop biological agents for the control of take-all of wheat which will bypass the years of severe disease prior to the natural onset of take-all decline.

Mechanisms implicated in take-all suppressive soils.

Mechanisms proposed to explain TAD have been grouped into two general categories: (i) microbiological changes in the soil or rhizosphere suppress the pathogen, and (ii) changes within the pathogen result in a loss of virulence. The former is the most widely held explanation. Based upon the original hypotheses of Cook and Rovira (1976) and Rovira and Wildermuth (1981), Weller (1983) suggested that TAD occurs because of a gradual build-up over years of antagonistic microorganisms on roots infected with *G. graminis* var. *tritici* and in infested root debris. Thus, active suppression by the antagonistic microflora occurs both

during the parasitic phase of *G. graminis* var. *tritici*, while the fungus is growing on the root, as well as during the saprophytic phase, while the fungus is in the debris and during growth toward roots of the next host crop (Weller 1983).

Although many genera of microorganisms have been implicated in TAD, the antibiotic-producing fluorescent *Pseudomonas* spp. have received much attention (Cook et al. 1995). Wheat roots from TAD soils supported significantly larger populations of fluorescent *Pseudomonas* spp. inhibitory to the take-all fungus than roots from take-all conducive soils (Weller et al. 1988), and *Pseudomonas* strains from TAD soils were significantly more effective at controlling take-all than those from conducive soils (Weller et al. 1985). Similar results were obtained by Sarniguet and colleagues (Sarniguet et al. 1992a, 1992b, Sarniguet and Lucas 1992). They demonstrated that remission of take-all on turfgrass was correlated with higher numbers of fluorescent pseudomonads (Sarniguet and Lucas 1992). Their results also suggested that qualitative changes in populations of fluorescent *Pseudomonas* spp. were more likely to explain different levels of soil conduciveness to take-all of wheat than were quantitative changes in this group of microorganisms (Sarniguet et al. 1992a, 1992b).

Various strains from TAD soils produced the antibiotic 2,4-diacetylphloroglucinol (PHL) (Keel et al. 1996, Raaijmakers et al. 1997) and some produced phenazine antibiotics (Thomashow and Weller 1988). Genetic studies, modeled after Koch's postulates, have demonstrated unequivocally that both PHL and phenazine antibiotics are key determinants of take-all suppression by some *Pseudomonas* strains (Keel et al. 1992, Thomashow and Weller 1988). Further, suppression of take-all of wheat was correlated respectively with the production in situ of phenazine-1-carboxylic acid (PCA) by *P. fluorescens* 2-79 (Thomashow et al. 1990) and with the production of PHL by *P. fluorescens* CHA0 (Keel et al. 1992). Collectively, these results led to the hypothesis that fluorescent *Pseudomonas* spp. producing phenazine antibiotics or PHL play key roles in take-all suppressive soils.

Phenazine antibiotics

Phenazine antibiotics are produced by various microorganisms including *Pseudomonas* and *Streptomyces* spp. and comprise a family of more than 50 pigmented, nitrogen-containing heterocyclic compounds probably derived from chorismic acid, a key branchpoint metabolite of the shikimic acid pathway (reviewed in Turner and Messenger 1986). Many phenazine antibiotics exhibit broad-spectrum activity against bacteria and fungi. Proposed modes of action include inhibition of RNA synthesis, the generation of cytotoxic superoxides and peroxides, or the disruption of membrane-associated metabolic processes such as active transport (Baron et al. 1989, Turner and Messenger 1986). Two of the most intensively studied phenazine antibiotics in relation to biological control of plant pathogens are PCA and 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA). PCA is a major determinant in the control of take-all of wheat by *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 (formerly *P. aureofaciens*) (Pierson and Thomashow 1992, Thomashow and Weller 1988). Both strains were isolated from roots of wheat grown in take-all suppressive soils from Washington and Kansas, respectively (W. Bockus *personal communication*, Weller and Cook 1983). Other phenazine-producing strains with biocontrol activity include *P. chlororaphis* PGS12, and *P. aeruginosa* strains LEC1, ln-b-109 and ln-b-784. Strain PGS12 was isolated from corn and selected on the basis of its activity in vitro against a variety of plant pathogenic fungi (Georgakopoulos et al. 1994). Production of the phenazine antibiotic pyocyanine contributes to the suppression of *Septoria tritici* by strain LEC1 (Flaishman et al. 1990). Strains ln1-b-109

and ln-b-784, which produce PCA and pyocyanine, are effective against the rice sheath blight pathogen *R. solani* AG1 (Rosales et al. 1995).

Phenazine biosynthetic loci have been cloned from *P. fluorescens* 2-79 (Mavrodi et al. 1996), *P. aureofaciens* 30-84 (Pierson and Thomashow 1992), and *P. chlororaphis* PGS12 (Georgakopoulos et al. 1994). The phenazine biosynthetic locus from *P. fluorescens* 2-79 is contained within a 12-kb fragment that was sufficient to transfer PCA biosynthetic capability to *E. coli* and to all of 27 recepient *Pseudomonas* strains into which it was introduced. DNA sequence analysis of the PCA biosynthetic locus of *P. fluorescens* 2-79 (Genbank accession number L48616) has revealed nine open reading frames (ORFs) designated *phzABCDEFG* (a single transcriptional unit), *phzR*, and *phzI*, spanning 8505 bp of the locus (Mavrodi et al. 1996, Mavrodi et al. *personal communication*). Four ORFs in the PCA locus of strain 2-79 encode predicted biosynthetic enzymes similar to those that function in aromatic amino acid synthesis. The regulatory genes *phzI* and *phzR* share sequence similarity with corresponding members of the *luxI/luxR* family, suggesting that regulation of PCA genes may be cell density dependent (Mavrodi et al. 1996, Mavrodi et al. *personal communication*).

2,4-Diacetylphloroglucinol

PHL inhibits a wide spectrum of fungi and bacteria, and is involved in biological control of many root and seedling diseases by fluorescent *Pseudomonas* spp. (Keel et al. 1992, Keel et al. 1996). These include suppression of black root rot of tobacco and take-all of wheat by *P. fluorescens* strain CHA0 (Keel et al. 1992, Stutz et al. 1986), suppression of Pythium damping-off of sugar beet by *Pseudomonas* spp. strain F113 from Ireland (Fenton et al. 1992, Shannon et al. 1992), and suppression of take-all of wheat by *P. fluorescens* strain Q2-87 (Harrison et al. 1993, Pierson and Weller 1994, Vincent et al. 1991) isolated from wheat grown in the Quincy TAD soil.

A 7-kb DNA fragment from *P. fluorescens* Q2-87 transferred PHL production to heterologous strains of *Pseudomonas* spp. (Bangera and Thomashow 1996, Hara et al. 1994). This fragment was analyzed by mutagenesis with the transposon Tn*3*HoHo1 to identify the portion required for synthesis of PHL (Bangera and Thomashow 1996). Insertions resulting in loss of PHL production spanned approximately 5 kb and delineated at least two divergently oriented transcriptional units. The DNA sequence was determined over this 5-kb region (Genbank accession number U41818) and six open reading frames (ORFs) were identified (Bangera and Thomashow 1996, Bangera and Thomashow *personal communication*). ORFs *phlA, phlC, phlB* and *phlD* encode the PHL biosynthesis enzymes and are transcribed as an operon from a promoter upstream of *phlA* (Bangera and Thomashow *personal communication*, Thomashow et al. 1996). The *phlD* gene product shares significant homology with members of the chalcone synthase/stilbene synthase family of enzymes from plants and is responsible for the synthesis of monoacetylphloroglucinol; a combination of PhlA, PhlC and PhlB is required to convert monoacetylphloroglucinol to PHL; *PhlE*, which is transcribed separately from *phlABCD*, encodes a product likely to be involved in processing, derivatization or export of PHL; and PhlF, which is divergently oriented from *phlACBD*, is a likely repressor of PHL production (Bangera and Thomashow *personal communication*).

Primers and probes to monitor phenazine and PHL producing *Pseudomonas* spp.

To further support the hypothesis that phenazine and/or PHL producing *Pseudomonas* play a key role in take-all suppressive soils, we developed specific primers and probes to detect these indigenous antibiotic-producing bacteria (Raaijmakers et al. 1997). Primers Phl2a and Phl2b were developed from sequences within *phlD*, which is responsible for synthesis of the PHL precursor monoacetylphloroglucinol. Primers Phl2a and Phl2b amplified the predicted 745-bp fragment from DNA of Q2-87 and a wide variety of PHL producing strains of worldwide origin. These results demonstrated that *phlD* is highly conserved among PHL producers confirming and extending the results obtained by Keel et al. (1996). Primers PCA2a and PCA3b were developed from sequences within the phenazine biosynthetic genes *phzC* and *phzD* from *P. fluorescens* 2-79. The phenazine primers amplified the predicted 1150-bp fragment from DNA of homologous strain 2-79 and allowed detection of various strains that produce PCA as well as other phenazine antibiotics that differ structurally from PCA. These results indicated that *phzC* and *phzD* are conserved among phenazine producing strains of *P. fluorescens, P. aureofaciens*, and *P. chlororaphis*.

The PHL probe was derived from the 745-bp fragment amplified from DNA of *P. fluorescens* strain Q2-87, whereas, the phenazine probe consisted of the 1150-bp fragment amplified from DNA of *P. fluorescens* strain 2-79. Both probes were used in colony hybridization and enabled specific and efficient detection of naturally occuring PHL and phenazine producing *Pseudomonas* strains (Raaijmakers et al. 1997). Colony hybridization was always followed by PCR as a quality check. The use of direct PCR is currently under investigation to provide a more rapid analysis of the presence of these antibiotic genes in environmental samples such as rhizosphere soil. It may also provide information about the existence of non-culturable microorganisms harboring these traits. The primers and probes allowed us to study the ecology of naturally occurring PHL and PCA producers in the rhizosphere of wheat plants grown in take-all suppressive soils.

Role of phenazine and PHL producing *Pseudomonas* spp. in take-all suppressive soils

The frequency of phenazine and PHL producing fluorescent *Pseudomonas* spp. was determined on roots of wheat grown in three take-all suppressive soils (TAD soils) and three take-all conducive soils collected from agricultural and virgin fields in Washington State, USA. (Raaijmakers et al. 1997). Wheat was grown in these soils for four successive cycles of four weeks each. This 'cultivation' of wheat was not intended to induce take-all suppressiveness, but merely to activate the resident microflora which includes the natural inoculum of the take-all fungus and its antagonists. The take-all fungus is naturally present in soils in the Pacific Northwest, USA. Surprisingly, phenazine producing fluorescent *Pseudomonas* spp. were not detected (detection limit was 10^4 CFU/g root) on roots of wheat grown in any of the soils. This was especially unexpected because phenazine producing strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 were isolated from take-all suppressive soils. Our results suggest that phenazine producing fluorescent *Pseudomonas* spp. are not enriched in the soils tested and consequently do not seem to contribute to the natural suppressiveness of these soils to take-all. In contrast, PHL producing fluorescent *Pseudomonas* spp. were isolated from all three TAD soils at densities ranging from approximately $5x10^5$ to $2x10^6$ CFU/g root but not from the corresponding take-all conducive

soils. These results suggest that PHL producing *Pseudomonas* spp. play an important role in the specific suppression that operates in take-all suppressive soils.

Concluding remarks

Evidence has been accumulating for the last two decades that antibiotic-producing fluorescent *Pseudomonas* spp. play a key role in soils that are naturally suppressive to take-all of wheat. Through the use of primers and probes directed against specific sequences within the PHL biosynthetic locus, we have demonstrated that populations of PHL producing fluorescent *Pseudomonas* spp. are highly enriched on roots of wheat grown in at least three different TAD soils but not on roots grown in the corresponding take-all conducive soils. We suggest that PHL-producers play a key role in the specific suppression that operates in take-all suppressive soils. Future work will focus on determining the role of these strains in TAD soils from across the USA. and around the world. It is notable that PHL-producing *Pseudomonas* spp. also have been readily isolated from soils suppressive to black root rot of tobacco and from soils suppressive to Fusarium wilt of tomato (Keel et al. 1996). For example, Keel et al. (1996) reported that 23% of the *Pseudomonas* isolates from roots of wheat grown in a black root rot suppressive soil contained PHL biosynthetic genes. Collectively, these results indicate the need for a detailed analysis of the population dynamics of PHL-producers in take-all and other natural disease-suppressive soils and to relate population sizes to disease incidence and severity. Primers and probes directed against specific genes involved in biological control may also expedite the search for strains that are better adapted to local soil conditions and more effective in specific crop-pathogen systems.

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Characterization of bacterial communities in the rhizosphere of *Chrysanthemum* using denaturing gradient gel electrophoresis and carbon utilization analyses

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Abstract

Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene segments was used to profile the dominant bacterial populations in the rhizosphere. Results showed a largely stable pattern for the bacterial communities in the rhizosphere in time and space. The patterns were similar to those produced with extracts obtained from bulk soil samples. Besides DGGE, single carbon utilization tests were used for randomly selected bacteria to profile the bacterial communities in the rhizosphere on metabolic activity. The variation in the fraction of randomly selected bacteria which could utilize a single carbon source was large between and within the samples. DGGE patterns obtained using microbial growth from dilution plates as the source of target DNA, were quite different from those found with the direct rhizosphere DNA extracts. Moreover, these patterns were sometimes different between the different replicate plates. This suggested that the plating medium used, selected only a limited number of bacterial species from the total populations under study. So the selection which was made using agar plates, is different each time and not representative of the dominant groups in the rhizosphere.

Introduction

Pathogenic *Pythium* species cause damage on crops and ornamental plants all over the world. Chrysanthemum, an economically important ornamental plant, is frequently plagued by *Pythium* infections, which cause root rot leading to lower branch weights of the plants. The oomycete *Pythium* is an avid colonizer of young roots and infects plants when they are stressed or when roots of the plants are damaged. Roots of plants are known to excrete several forms of organic materials. The amount and composition of the organic materials differ between plant species and between plant cultivars, as well as during the development of a plant and between different places on the root system (Lynch and Whipps 1990). Bacterial populations, which utilize the organic materials as substrate may vary in composition and density during the development of the plant (Curl and Truelove 1986). This may hold for both saprophytes and pathogens. The root pathogen *Pythium* utilizes the root excreted organic materials as substrate and as a signal to find and infect the root (Martin 1995). In order to find suitable antagonists against *Pythium*, we search for bacteria which are able to compete for the root exudates at the right time and at the right place in the rhizosphere, i.e., the place and time of infection by *Pythium*. For that reason, we aim to analyze the bacterial communities in the rhizosphere during plant development.

Currently, molecular methods are used in several fields of ecological research. In microbial ecology these methods can give information about population structures of microorganisms, both culturable and non-culturable. In the field of biological control where we try to find antagonists of bacterial origin, beside the molecular methods, we still need isolation procedures on agar because antagonists should eventually be culturable. Here we show the results of community analyses of microflora using a molecular method by which 16S ribosomal RNA based PCR is followed by denaturing gradient gel electrophoresis (DGGE) and carbon utilization analyses of randomly collected bacterial isolates on agar plates.

Materials and Methods

Chrysanthemum plants were grown in Ede loamy sand soil, in a growth room (20 °C day and night temperature, 70% RH and 16:8 light:dark photoperiod). Three weeks after placing the plants in the soil, day length was changed from 16 to 8 hours to induce the flowering period. Root samples with adhering soil were collected from two, four, six, eight and ten week old plants. Samples of young root parts (1 to 2 days old) and old root parts, 1 and 4 cm in length respectively, were collected.

For the DNA-analyses of the bacterial communities, two plants were used at each sampling date. Beside the rhizosphere also samples of bulk soil were collected. DNA extraction and purification from the bulk soil and rhizosphere samples was performed using the method of Smalla et al. (1993). PCR amplifications were performed with the (clamped) F968 and R1401 universal bacterial primers. PCR products, of around 450 bp, were analyzed on conventional agarose gels. The present method discriminates only bacterial populations which comprise 1% or more of the total bacterial communities and which can therefore be considered to be a dominant group. Denaturing gradients of various steepness were prepared in accordance with Muyzer et al. (1993). Gradients between 50 and 65% of denaturant commonly produced optimal separation of PCR generated bands and were routinely used. Samples of 20 ml of PCR product were loaded on gel, after which gels were run for 6 or 16 h in an Ingeny DGGE setup at 60 °C. Gels were stained with Sybrgreen, after which they were inspected under UV and photographed.

Root samples of six plants, were used to analyze the diversity of culturable bacterial species. Per sample, thirty bacteria isolated on 0.1 TSA agar, a general medium, were characterized by testing their ability to grow on different single carbon sources. Simmons' citrate agar was used in this study as the basic medium (Gerhardt 1981) to carry out the carbon utilization tests. The single carbon source citrate (1 g/liter) was replaced by chitin, starch, sucrose, maltose, cellobiose, fructose, xylose, glucose, galactose, fucose, sodium oxalate, sodium succinate, alanine, glutamine acid, glutamine, serine, phenylalanine and proline, respectively. The colonies of bacteria of two samples, grown on 0.1 TSA agar plates, were analyzed by the DGGE method.

Results and Discussion

The rhizosphere samples collected from different root sites during plant development showed little variation in the banding pattern when analyzed with DGGE (Fig. 1). Only a few extra bands appeared for samples taken at the end of the growing period of chrysanthemum. This means that the dominant groups in the rhizosphere are rather stable during plant development for tip and base. Differences were small between replicates. To assess whether the rhizosphere induces shifts in the dominant bacterial groups of soil, samples of bulk soil were also analyzed. The data indicated only a few differences between samples taken from rhizosphere

and bulk soil (Fig. 2). This indicates little effects from the plant root or the root-excreted material on the dominant bacterial groups in soil. There can still be an influence from the root on specific bacterial populations, but these organisms obviously do not belong to the dominant groups. When the DNA of direct extraction is compared with DNA of plates using DGGE (Fig. 3) only a few similarities were found. This means that bacteria which are culturable and grow on 0.1 TSA do not necessarily represent the groups that appear to be dominant in the rhizosphere. Replicates of agar plates of one sample (Fig. 3) resulted in several different bands. This means isolation on agar plates results in a random selection of soil bacteria.

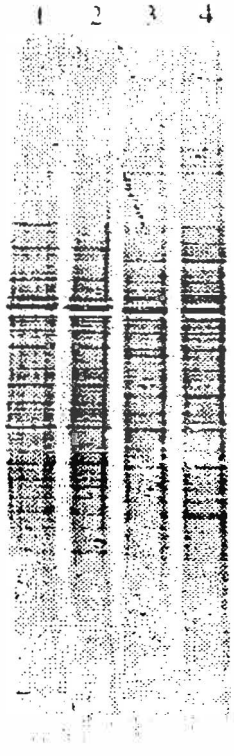

Fig. 1. DGGE profiles of 16S rRNA-defined bacterial populations. The samples are from root parts of one to two days old roots with adhering soil collected from chrysanthemum plants after the second week (1 and 2) and eight week (3 and 4) of growth of the plants.

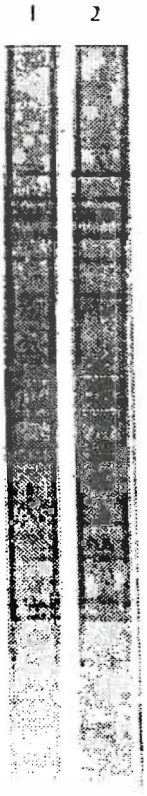

Fig. 2. DGGE profiles of 16S rRNA-defined bacterial populations. The samples are collected from collected from bulk soil (1) and rhizosphere soil (2).

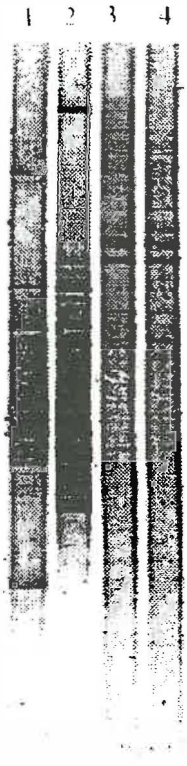

Fig. 3. DGGE profiles of 16S rRNA-defined bacterial populations. Profiles were obtained from direct DNA-extraction of soil (3 and 4) and profiles are from culturable bacteria which were able to grow on 0.1 TSA plates (1 and 2).

In the carbon utilization tests, the active and culturable bacteria were analyzed. The way in which randomly selected bacteria reacted on the carbon sources showed much variation between and within samples. When the results for the four main groups of the carbon sources are summarized, there is an apparent trend after the fourth week of growth of the chrysanthemums. The fraction of bacteria isolated from the tip that can utilize organic acids, amino acids and simple sugars is at maximum (Fig. 4). In the third week of plant growth the light period was changed from 16 h light to 8 h light to induce the flowering period. This is also a period when plants are extra vulnerable to *Pythium*, since it is a moment of increased root turnover. The summarized results for the base show a trend in which the number of bacteria which could use complex sugars increased during the development of the plant (Fig. 4). This indicates a response to increased cell lysis at the base, by which more complex organic materials are released. When the reaction on the single carbon source was analyzed separately, the trends just mentioned were not always visible. Perhaps an other set of single carbon sources could have been more discriminated between the treatments and give less variation within the treatments (Hitz et al. 1997). However, the carbon sources were chosen in such a way, that they fit the carbon utilization pattern of *Pythium*. On the other hand a random sample survey of 30 isolates is possible too small to be representive for the sample.

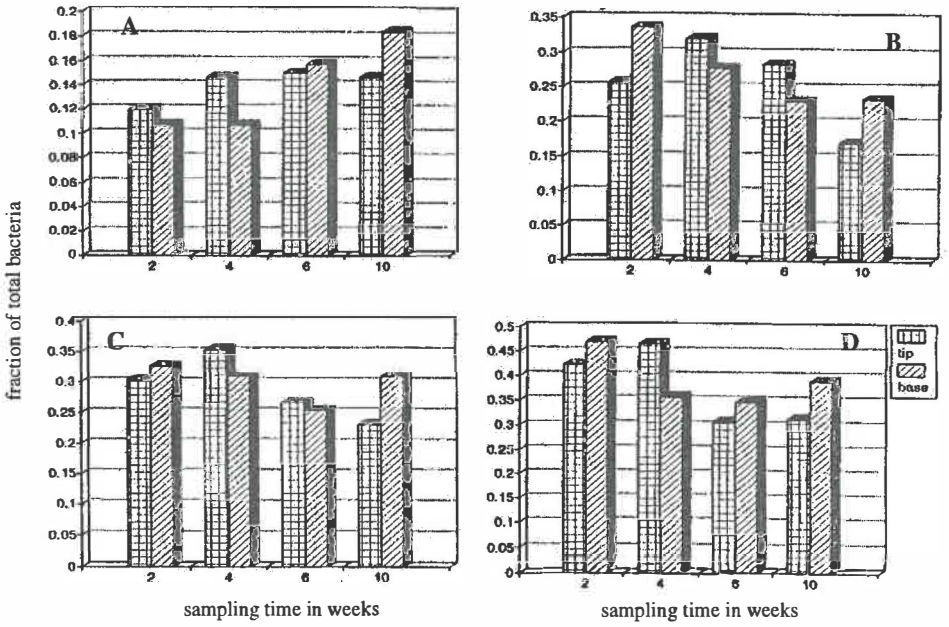

Fig. 4. Fraction of bacteria selected from the rhizosphere of chrysanthemum which can use a single carbon source. Complex sugars (A) consists of chitin, starch, sucrose, maltose and cellobiose. Simple sugars (B) consists of fructose, xylose, glucose, galactose and fucose. Organic acids (C) consists of oxalate and succinate. Amino acids (D) consists of alanine, serine, glutamine, glutamine acid, phenylalanine and proline. Data are presented as the average of the single carbon sources which belong to a, b, c and d, respectively. Data were obtained from six plants.

The results of the agar plates analyzed with DGGE, which show that bacterial colonies grown on agar plates do not represent the dominant groups, explains the differences found for the two methods used in this study and the differences between DGGE and information from literature (Curl and Truelove 1986). The results of DGGE of direct extraction show that dominant bacterial groups in the soil are very stable, both in and outside of the vicinity of roots, which is contrary to the general thoughts on the rhizosphere effects. With these results we should realize that selected isolates from agar plates with good properties to be an possible antagonist, may not belong to a dominant group in the rhizosphere and are introduced in a biologically stable environment. This can explain the difficulties that are reported on the effectiveness of soil inoculation with antagonistic bacteria. Yet, we are dependent on isolation using agar plates or other selective media to obtain suitable antagonists against root pathogens. A combination of both methods should be used to select possible antagonists. In future work DGGE with RNA should be conducted as well, since this method will only show the dominant bacteria groups in the rhizosphere which are active.

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Applications of the GUS marker for autecological investigations of *Trichoderma harzianum* in complex natural environments

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Abstract

Ideally, methods for application of a biocontrol agent should ensure that the antagonist is present and active at the right place at the right time, namely at the specific microsites where the pathogen is found, and where the antagonist consequently has to work. This being so, a fundamental understanding of the autecology of the antagonist is essential for improvement of biocontrol methods. In this connection, the GUS marker has proven to be a useful tool for monitoring an introduced strain of *Trichoderma harzianum* in complex environments. Three distinct methodologies are presented, each enabling investigation of a different ecological aspect of *T. harzianum*. One method is useful when studying spread and survival of an introduced strain in soil and soilless potting mixtures. Another can be used as a means to determine the general metabolic activity of the antagonist in specific habitats. The final method facilitates in situ localization of metabolically active hyphae of the transformant.

Introduction

The fungal antagonist *Trichoderma harzianum* Rifai has proven to be an effective biocontrol agent against a range of soilborne plant pathogens both when applied as a seed treatment or when mixed into soil and soilless potting mixtures (Chet 1987, Jensen and Wolffhechel 1995, Papavizas 1985). Like most biocontrol agents, *Trichoderma* spp. are influenced to a much greater degree by the environment than agrochemicals. The effectiveness by which they are able to control disease is very dependent on abiotic and biotic factors relating to the micro-environments in which the pathogen is found, and where the antagonist consequently has to work. This being so, understanding the ecology of the antagonist is critical for improvement of biocontrol methods and for the practical implementation. But until now very little has been published on the ecology of *Trichoderma* spp. Even the modes of action in vivo remain in doubt as they do not necessarily correspond to the reported modes of action in vitro. However, studies on fungal autecology in natural soil and organic growth substrates have been particularly difficult to carry out because of the microscopic size of the ecological niches and the extreme biotic diversity of the various habitats. Recent progress in the development of molecular methods is enabling autecological investigations in complex natural environments to be carried out.

This paper discuss the use of the β-glucuronidase gene (GUS) as a marker for monitoring an introduced strain of *Trichoderma harzianum* in complex environments. The discussion is related to general methodologies for assessing microbial populations, biomasses, and activity in soils.

Phenotypical resemblence of transformant and wild-type

Thrane et al. (1995) co-transformed *T. harzianum* with the *Escherichia coli* β-glucuronidase gene (GUS) and the *E. coli* hygromycin resistance gene (Hyg B) as the selective marker. Both genes were inserted behind the constitutive *Aspergillus nidulans gpd* promoter. The transformants were mitotically stabilized and insertion of the genes into the chromosomal DNA verified. In order to find one isolate that phenotypically resembled the wild-tpye, three transformed isolates were examined in vitro for their physiological similarities to the wild-type (Thrane et al. 1995). These examinations included: morphology, growth rate, dual culture with the wild-type and with *Pythium ultimum*, conidial germination, glucose utilization, secondary metabolite and extracellular protein profiles. One of the transformants, T3a, that in all tests resembled the wild-type was then tested for its fitness in the rhizosphere of cucumber plants grown in steamed sphagnum peat (Green and Jensen 1995). Population studies, carried out by dilution plating on a selective medium, revealed that the transformant grew and increased in the rhizosphere just like the wild-type (Green and Jensen 1995). This was the final confirmation of the phenotypical resemblance of transformant and wild-type, thus allowing the transformant to be used for reliable ecological studies. The importance of comparing both the physiological properties and the ecological fitness of the transformant with the wild-type can not be over-emphasized. When doing ecological studies with a transformant it should ideally only differ from the wild-type, which is the actual organism of interest, in terms of the gene insert and any phenotypical alteration caused by its product. In all other respects, the wild-type and transformant should be both genotypically and phenotypically similar. Examining profiles of primary and secondary metabolites in vitro are examples of such important physiological tests. Similarity in these respects also adds to the probability that the fitness of the transformant resemble the wild-type under different ecological conditions.

Soil dilution plate assay

In the early period of soil microbiology the soil dilution plate technique was used almost exclusively for both quantitative and qualitative studies of soil fungi (Parkinson and Coleman 1991). The method has been much discredited, one of the major criticisms being that the majority of colonies arise from conidia rather than hyphae (Warcup 1955). As such, the propagules which are counted are not necessarily those which are functioning in the ecosystem (Brock 1987). However, in soils nutrient availability is a limiting factor for microbial growth and activity. Consequently, most microorganisms exist in a state of exogenous dormancy or fungistasis (Lockwood 1977). Even in the case where easily accessable nutrients have been released due to steaming of sphagnum peat (Bollen 1974), actual biomass estimation of *T. harzianum* based on FDA vital staining (Söderström 1977) and subsequent measuring of hyphal length, has not been possible (Green and Jensen 1995). This is because the cycle from germination of conidia to formation of conidiophores with new conidia is very fast with restricted hyphal growth in between. According to Eastburn and Butler (1988), *T. harzianum* falls into the category of ruderal fungi, which generally do not grow through soil, but form sporulating colonies near the surface of the colonized substrates (Pugh 1980). Apart from growing on colonized plant debris or other suitable food bases, *Trichoderma* spp. will consequently exist in soil or soilless potting mixture mostly as conidia. Therefore, when studying spread and survival or in comparative studies of an introduced strain and indigenous species, a combination of a marked strain and dilution plating on

selective media, seems to be a reasonable choice for monitoring the antagonist. The problems with background microflora, which even on selective media has been a major concern when carrying out isolation from complex environments, can thus be eliminated. We successfully applied dilution plating on *Trichoderma*-selective medium combined with a simple test for GUS activity of the colonies to compare the population development of the wild-type and the transformant in the rhizosphere of cucumber seedlings grown in peat-based potting mixture infested with both organisms (Green and Jensen 1995).

Chemical determination of biomass and activity

When studying ecology and function of micro-organisms in specific niches like the rhizosphere or on plant debris, the major requirements are to measure changes in biomass and metabolic activities of the individual species. With the limitations of using colony-forming units (CFU) in quantitative and qualitative investigating of soil fungi, many microbial ecologists have considered chemical determination an attractive possibility for biomass and activity estimation, but most of the substances chosen in this context do not distinguish single species from the total microbial population. However, the integration of marker genes into the genome of micro-organisms enables the production of compounds with the necessary degree of specificity. The GUS marker seems very promising for use in ecological studies because background activity is, with some exceptions, either absent or low in plants, fungi, and bacteria investigated (Jefferson 1987, Wilson et al. 1991). Furthermore, the enzyme is fairly stable and can easily be assayed by different methods (Gallagher 1992, Jefferson 1987). GUS transformants of some plant pathogenic fungi have been used for detection and biomass quantification in infected plant tissue (Couteaudier et al. 1993, Köhler et al. 1995, Liljeroth et al. 1993, Oliver et al. 1993).

Generally, the enzymes encoded for by gene inserts, are not secreted by eucaryotic organisms unless the inserts are constructed with special signal sequences that allow them to be transported from the endoplasmatic reticulum through the Golgi apparatus to the cell surface. Therefore, the specific enzymes are only released into the soil when the cells disintegrate either due to natural death of (part of) the transformants or due to a physical destruction in connection with the extraction procedure. Enzymes released into the soil due to natural death of the organisms are absorbed onto organic and mineral constituents or complexed with humic substances, or both. The amount of free enzymes in the soil solution is therefore minute compared to that in the absorbed state (Tabatabai and Fu 1992). In other words, the enzymes are either associated with soil constituents or the living biomass. This knowledge implies that by removing organic and mineral soil constituents from the extraction buffer immediately after physical destruction of mycelium and spores, it should be possible to measure the activity of the enzymes, which just prior to extraction, were associated with the living biomass of the transformants. In this way we have monitored the GUS activity in peat-based potting mixture infested with the transformed strain of *T. harzianum*, T3a (Green and Jensen 1995). The fact that the level of GUS activity strongly correlated with the presence of active and germinating conidia in the peat measured by fluorescence microscopy supports the hypothesis that the amount of GUS, which was present in the assay solution, was that which had been extracted from the living biomass of the fungus. However, as the gene is controlled by the constitutive *Aspergillus nidulans gpd* promoter, the GUS activity rather than being an indication of the biomass, qualifies as an expression of the general activity of the fungus (Green and Jensen 1995). In accordance with this, Eparvier and Alabouvette (1994)

considered a constitutively expressed GUS gene in *Fusarium oxysporum* f.sp. *lini* to reflect the metabolic activity of the strain. While these approaches makes it possible to study fungal activity in complex systems by extraction and quantification of GUS, one should be aware that in nonsterile soil, some background activity may occur due to the presence of bacteria or fungi with intrinsic GUS activity.

In situ detection

In terms of biological control it is important that the antagonist is present and active at the specific microsites where the pathogen is found and where the antagonist consequently has to work. As such, to control root diseases, an antagonist should ideally possess the ability to colonize potential infection sites along the rhizoplane despite competition from other microorganisms. Until recently, studies of root colonization by *Trichoderma* spp. have been based on indirect techniques such as determination of the number of colony-forming units (CFU) per centimeter of root. However, this technique mainly detects the presence of conidia on the root surface and does not, therefore, reflect the actual activity of the fungi (Green, Heiberg, and Jensen, *unpublished data*). Lately, the integration of marker genes into fungal genomes has allowed direct detection of hyphae in association with plant tissue either internally or externally. By use of the synthetic substrate 5-bromo-4-chloro-3-incolyl-β-D-glucuronide (X-gluc), metabolically active hyphae of a GUS transgenic fungus turns blue or turquoise and becomes clearly visible with the naked eye or with various microscopic techniques.

Eparvier and Alabouvette (1994) used GUS transgenic strains to examine competition between pathogenic and nonpathogenic *F. oxysporum* on roots. With this technique, it was clearly shown that the activity of both the pathogenic and the non-pathogenic strain was reduced in the presence of the other, and that competition for actual infection sites was taking place. By use of the GUS transformed strain of *T. harzianum* we have obtained results suggesting that exudation from healthy roots is not sufficient to support any activity of the antagonist. However, several different niches that support growth and activity of *T. harzianum* in the spermosphere and rhizosphere were identified (Green, Heiberg, and Jensen, *unpublished data*). These include the seed coat, wounds, infected tissue, and dying rootlets. Thus, in such special niches, saprophytic organisms like *T. harzianum* will be well adapted, capable of utilizing their antagonistic mechanisms (i.e., antibiosis) to facilitate competition for nutrients and sites.

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Set-up of a biocontrol experiment with genetically characterized hypovirus-infected *Cryphonectria parasitica* strains

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Abstract

For a better understanding of its dissemination, the *Cryphonectria* hypovirus has been artificially introduced into two different *C. parasitica* populations. Chestnut blight cankers were treated with a hypovirus-infected strain. In one population (Choëx, Valais) hypovirulence was absent before the treatment, whereas in the other (Claro, Ticino) it was already naturally present. The aim of the experiment was to track the introduced virus as well as its fungal carrier and to survey the dynamic of the disease in these stands. In Choëx, where no other viruses were present, the virus is easily detected by verifying the virus-associated culture morphology of the isolated strains. In Claro, RFLPs of two fragments amplified from the reverse transcribed viral genome (RT-PCR) serve as markers to distinguish the introduced virus from the resident hypoviruses. Similarly, a DNA fingerprinting method is used to identify the introduced fungal strain.

Introduction

*Cryphonectria (*Syn. *Endothia) parasitica* (Murill) Barr., an ascomycete, is the causal agent of chestnut blight. After the introduction into the eastern USA at the beginning of this century it virtually eliminated the American chestnut trees (*Castanea dentata*) in their natural range (Anagnostakis 1987). A similar result was anticipated when chestnut blight was found in Europe near Genova (Italy) in 1938. However, the unexpected appearance of transmissible hypovirulence prevented the European chestnut (*C. sativa*) from succumbing to the blight (reviewed by Heiniger and Rigling 1994).

Hypovirulence is caused by an unencapsidated double-stranded (ds) RNA virus, which is transferred between fungal strains via hyphal anastomosis (Choi and Nuss 1992, Day et al. 1977, Nuss 1992). Infection by the hypovirus causes a reduction of fungal virulence (hypovirulence) ranging from avirulence to almost normal virulence (Bazzigher et al. 1981, Rigling et al. 1989). Furthermore, infected strains show reduced sporulation and pigmentation, resulting in the typical white cultural appearance.

The hypovirus is of great interest for biological control. However, the exact mechanisms of its dissemination are still unclear. Several factors restrict the dissemination of the hypovirus in fungal populations: i) vertical transmission occurs only into asexually produced conidia, but not into sexually produced ascospores, ii) the rate of transmission into conidia varies considerably, iii) conidia production is reduced in hypovirulent strains and iv) a system of vegetative incompatibility limits horizontal transmission of the virus among fungal strains (Anagnostakis 1988, Liu and Milgroom 1996). Nevertheless, the hypovirus is disseminated quite efficiently in many European *C. parasitica* populations.

For a better understanding of the dissemination of the hypovirus, an experiment in two different plots was started. Biocontrol treatments with selected hypovirus-infected strains were performed. Subsequently, the temporal and spatial movement of the introduced hypovirus and its fungal carrier will be followed by using genetic markers.

Materials and Methods

The two experimental plots in Choëx and Claro are *C. sativa* coppice stands. Choëx is located at the edge of the occurence of chestnut blight in Switzerland. The disease was first recorded in this region in 1986 (Heiniger and Stadler 1990). Claro is situated in southern Switzerland, where chestnut blight has been present since at least 1948 (Gäumann 1951).

Isolations of *C. parasitica* were performed as described in Bissegger et al. (1997), with the modification that tannic acid malt extract agar (Rigling 1995) was used as isolation medium. The isolates were assigned to vegetative compatibility groups (VCGs) according to the merging/barrage response (Anagnostakis 1988). Culture morphology and dsRNA extractions were used to identify hypovirus-infected isolates. The inoculum source had to be prepared after selecting a hypovirus (the same for both plots) and fungal strains (one for each plot) adapted to the resident populations. The selected fungal strains (carriers) were infected with the hypovirus by pairing them in vitro with a strain infected with the selected hypovirus. The hypovirulent inoculum for the biocontrol treatment was grown in liquid culture with EP complete medium (Day et al. 1977) at 25 °C for 5 days. Cankers were treated by making holes at the margin of the infection using a cork borer (5 mm in diameter). The holes were filled with the inoculum and covered with tape.

RFLPs of two fragments amplified from the reverse transcribed viral genome were used to identify the introduced hypovirus. dsRNA was isolated by cellulose chromatography (Morris and Dodds 1979). First strand cDNA was synthesized from the purified dsRNA using random primers. The cDNA was used as template to amplify two fragments (one from ORF A and one from ORF B) of the viral genome. These fragments were digested separately with different four-cutter restriction enzymes. RFLPs were visualized by running the digests on agarose gels. Similarly, a DNA fingerprinting method developed by Milgroom et al. (1992) was used to identify the introduced fungal strain. This method consists of probing Southern blots of *Pst*I-digested total DNA with a multilocus probe (pMS5.1). Instead of radioactivity, DIG-labelling and detection by chemiluminescence was used.

Results and Discussion

The fungal population in Choëx was very clonal. Only one vegetative compatibility group (VCG) was found among 140 isolates tested. The same VCG was found among 23 isolates from the surrounding area. Almost all DNA fingerprints were identical. Only 3 out of 56 isolates showed a slightly different pattern. This can be explained by the recent introduction of the disease in this region (Heiniger and Stadler 1990). Most probably, only a very few genotypes were responsible for the infection in Choëx. Furthermore, no hypovirus-infected strains were isolated, i.e. no hypoviruses were present. The presence of only one VCG in this plot greatly favors the application of biocontrol treatments. One single hypovirus-infected strain has the potential to convert all other strains in the plot, thus rising the prospects of a good establishment of hypovirulence. The clonal fungal population without any hypovirus infection also facilitates the tracking of both, the hypovirus and its fungal carrier. The

introduced hypovirus can easily be detected by verifying the virus-associated culture morphology of the isolated strains. A fungal strain was selected from the culture collection (at the Swiss Federal Institute for Forest, Snow and Landscape Research, CH-8903 Birmensdorf) that fulfilled the criteria shown in Table 1.

Table 1. Criteria for the selection of the hypovirus and its fungal carrier for the biocontrol experiment

Hypovirus		
	Virulence	The hypovirus should not deblilitate its fungal host too much. The hypovirus-infected strain must still be able to form cankers and to produce asexual spores.
	Identity	The hypovirus must be genetically distinguishable from the resident viruses (RT-PCR/RFLP).
Fungal strain		
	VCG	The fungal strain must be in the dominating VCG of the plot. This is crucial for the horizontal transmission of the hypovirus and prevents emergence of new VCGs by outcrossing.
	Identity	The fungal strain must be genetically distinguishable from the resident strains (DNA fingerprinting).

The fungal population in Claro shows a completely different picture compared to Choëx. Twelve different VCGs were detected among 170 isolates tested. More than 90% of the isolates could be assigned to only four major VCGs. Similarly, a high diversity in DNA fingerprints was detected. Fourty different patterns were observed among 58 isolates. Almost 50% of the isolates were hypovirus-infected. Comparable diversity was found in other populations in southern Switzerland (Bissegger et al. 1997). Therefore, for subsequent tracking in the field, the hypovirus and the fungal strain had to be selected carefully according the criteria shown in Table 1.

Biocontrol treatments were performed in order to introduce the hypovirus-infected strains in the plots in fall 1996. In Choëx about 30% and in Claro about 20% of the cankers were treated. In spring 1997, a subset of the treated cankers was sampled to determine if the virus was still present after the winter period. Strains exhibiting the white culture morphology could be isolated from all of the sampled cankers, indicating a good establishment of the introduced hypovirus. The temporal and spatial movement of the hypovirus and its fungal carrier will now be followed by examining the population periodically. The results of this study will lead to a better understanding of the dissemination of the *Cryphonectria* hypovirus and thus be helpful to current attempts for a sustainable biological control of chestnut blight.

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Mycelial growth of pathogenic and nonpathogenic *Fusarium oxysporum* strains in the vicinity of roots

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Abstract

The patterns of mycelial development (length and branching) of four pathogenic strains of *Fusarium oxysporum* (two formae speciales *lycopersici* and two f.sp *radicis-lycopersici*) have been compared to a nonpathogenic strain currently used as a biocontrol agent. Our objective was to determine whether the pathogens or the biocontrol agent were preferentially stimulated in the vicinity of the roots of both the host plant (tomato) and a non-host plant (wheat). Hyphal extension and branching were measured microscopically using a CCD camera and an image analyzer. The role of soluble root exudates, insoluble root materials, glucose supply, and nitrogen supply (organic forms) on mycelial development has been investigated. Each of the strain had its own pattern of mycelial development in the absence of root. A high level of stimulation of the fungal development was found for the 5 strains in the vicinity of both the tomato roots and the wheat roots but this stimulation was not related to the pathogenicity of the strains. The presence of organic nitrogen in the soluble exudates was mainly responsible for this stimulation. Therefore, it is likely that the recognition mechanisms between the host plant and the pathogenic or nonpathogenic strains occur within the root. Outside of the root, both pathogenic and nonpathogenic *F. oxysporum* are similar populations with their own development features which are not related to their pathogenic traits.

Introduction

The *Fusarium oxysporum* species includes numerous plant pathogenic strains causing either wilt disease or crown and root rots on a broad range of agricultural and ornamental host plant species. The pathogenic strains are each regarded as specialized parasites of limited host range and are grouped together into *formae speciales* according to the host plant species (Armstrong and Armstrong 1981). The species also includes nonpathogenic strains. Although they are not pathogenic towards host plants these strains are very aggressive colonizers of the plant rhizosphere and plant roots but they do not induce any symptoms and do not have any apparent detrimental effects on the colonized plant (Burgess 1981). The nonpathogenic populations are able to limit disease incidence in naturally suppressive soils and in conducive soils and soilless cultures when inoculated as biocontrol agents (Alabouvette et al. 1993, Minuto et al. 1995, Paulitz et al. 1987). Olivain and Alabouvette (1997) observed that nonpathogenic strains were able to build up a dense mycelial network around the root. This network could prevent the host plant from penetration by a pathogenic strain. Therefore it could be interesting to test whether the mycelial development of pathogenic or nonpathogenic strain could be preferentially stimulated in the root vicinity of the host plant. Recently, Edel et al. (1997) showed that some *F. oxysporum* populations became dominant in the root system of plants while they were not in the soil. The populations which became dominant were

depending on the plant species suggesting a particular relationship between the plant and *F. oxysporum* populations. Although these populations were nonpathogenic populations of *F. oxysporum* (Steinberg et al. 1997), one may question whether a host plant could modify the behavior of either pathogenic or nonpathogenic strains because the rhizosphere competence of *F. oxysporum* populations in relation to pathogenicity has not yet been investigated.

The objectives of this study were to compare the development of five strains of *F. oxysporum* in the vicinity of the roots of an host plant in relation to their pathogenicity, and to determine what could be responsible for the stimulation of fungal development.

Materials and Methods

Five strains of *F. oxysporum* were used: strain Fo47 is a nonpathogenic strain; strain Fol8 and strain Fol15 are responsible for wilt disease on tomato and therefore belong to the f.sp. *lycopersici*, while strain Forl19 and strain Forl22 are responsible for crown and root rot of tomato and therefore belong to the f.sp. *radicis-lycopersici*. The strains were cryo-preserved in liquid nitrogen and were subcultured on potato dextrose agar (PDA) at 25 °C. Liquid subcultures were performed at 25 °C in synthetic mineral medium (MM) plus glucose (5 g/liter) (Correl et al. 1987).

Two plant species were used; tomato (*Lycopersicon esculentum* Mill. cv. H63.5) which is susceptible to Fusarium wilt, and wheat (*Triticum vulgare* cv. Ventura) which is not susceptible to any disease caused by *F. oxysporum*.

Seeds were surface sterilized using the method of Speakman and Krigger (1983). Plants were grown in a sterile plant nutrient solution (Bennett and Lynch 1982). Roots were aseptically collected from 7 to 8 day old seedlings. Root tips (3 to 4 cm long) were rinsed in sterile distilled water. Some were directly used for the microscopic measurements and the others were freeze dried.

Root soluble components were separated from the root insoluble materials by boiling the freeze dried root tips under reflux for three consecutive 15 min in three changes (approx. 20 ml) of 80% ethanol. The three EtOH extracts were pooled and the extraction solvent was evaporated using a rotary evaporator (waterbath 60 °C). The soluble components were recovered in sterile distilled water, filter sterilized (2 µm pore size), aliquoted in eppendorf tubes, and freezed. The insoluble components were freeze dried. The basic structure of the root was preserved by the treatment, and the frozen root tips could be handled in the same way as fresh tips.

The microscopic measurements were performed on individual fungi growing on a film of agar, on a coverslip placed in a humid chamber. Depending on the experiments, the film consisted in MMA (MM plus agar 15 g/liter), MMAG (MMA plus glucose) or MMAN (MMA plus Bacto casamino acids), MMAGN (MMA plus glucose plus casamino acids, ratio 4:1). The glucose concentrations used were 0.1, 0.5, 1, 5, 10 g/liter. The casamino acids concentrations used were 0.025, 0.125, 0.25, 1.25, 2.5 g/liter.

A suspension of conidia originating from a 24 h liquid culture was adjusted to $5x10^3$ conidia/ml. Fifty milliliters of this suspension were then inoculated on the top side of the coverslip and allowed to uniformly cover the surface of the agar film by gentle shaking.

When roots were used, two root tips were put on the MMA film. Then the inoculation was performed. To test the role of root exudates on the fungal development, two aseptically rinsed fresh roots per coverslip were placed on the MMA as above. The petri dishes were allowed to incubate for 18 h at 25 °C, then, the root tips were removed and inoculation was

performed. To test the role of EtOH soluble extracts, a volume corresponding to the soluble extracts of two roots was deposited on the MMA film. The plates were allowed to incubate for 30 min to ensure a complete incorporation of the extract in the MMA film and inoculation was performed.

Ten petri dishes were prepared per strain and per treatment. The petri dishes were allowed to incubate upside down at 25 °C until the microscopic measurements. (Zeiss, 10x). A CCD camera (Ikegam, Japan) and an interactive image analyzer (Vids V, Synoptics Ltd.) were used to record the data. At least 15 individual fungi per strain and per treatment were measured at each time of the kinetics. Kinetics consisted of three times of measurement (14, 16, 19 h) when five strains were used, and five times of measurement (14, 16, 18, 20, 22 h) when three strains were used. When no root was used individual fungi were randomly taken on the surface of the film. When roots were used, only individuals found within a distance of 1.26 mm from the root and not in contact with it were measured. This distance corresponds to a microscopic field and will be referred as the root vicinity.

Analyses of variance, performed using Genstat® were used to test for significant differences ($P = 0.05$) between the mycelial development of the five strains and to determine whether a treatment stimulated one of the parameter for each of strain. Newman-Keuls multiple range test was used to compare treatment means.

Results and Discussion

When inoculated onto MMA with no root, the five strains were able to germinate, to elongate and to branch but significantly different lengths were achieved by the strains (Table 1). Strain Fol15 has a significantly higher number of ramifications than the four other strains and Forl19 had a significantly higher branch mean length. The hyphal length, the number of branches and the branch mean length significantly increased with time but for each of these parameters, a significant interaction strain time was found. These interactions indicate that the variations were different from strain to strain for each parameter.

Table 1. Means of the lengths, numbers of branches and branch mean lengths achieved by the five *F. oxysporum* strains on MMA. Values with the same letter within a column are not significantly different ($P = 0.05$).

Strain	L (length, mm)	N (number of branches)	L/N (branch mean length, mm)
Fo47	506.7 d	2.5 b	190.4 b
Fol8	701.3 c	2.8 b	233.8 b
Fol15	1101.4 a	5.4 a	199.2 b
Forl19	936.9 b	3.0 b	372.7 a
Forl22	656.4 c	2.7 b	223.3 b

Therefore, because the anova indicated (i) that the hyphal lengths and the branching achieved by the strains were different, and (ii) that the speed of development was varying within strains, it can be assumed that mycelial development is a specific trait for each strain and this trait is not connected to pathogenicity. Indeed, the two strains with similar hyphal length are Fol8 and Forl22 and these two strains are responsible for two different types of Fusarium disease.

When the strains were inoculated in the root vicinity, both total hyphal length, number of branches and branch mean length were significantly increased for all the strains. An example is given in Fig. 1 for strain Fo47. This result indicates a rhizosphere effect. The rhizosphere effect has been assessed on propagules density but not on mycelial growth. To evaluate whether such a stimulation was strain specific or not, ratios were calculated for all the parameters:

mean of a parameter for 15 individuals in the treatment/
mean of the parameter for 15 individuals on MMA.

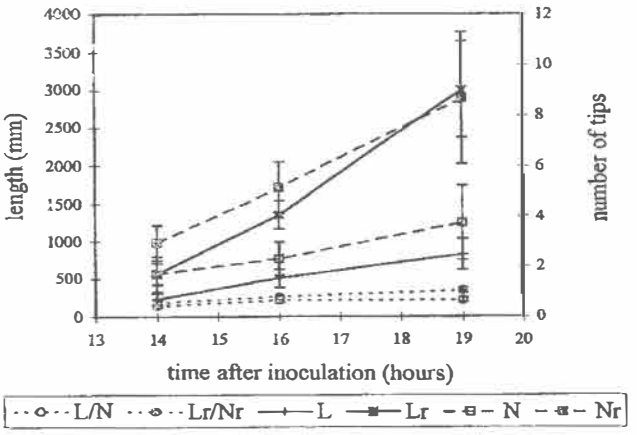

Fig. 1. Kinetics of development of the strain Fo47 on MMA and in the vicinity of tomato roots. L: Hyphal length on MMA, Lr: Hyphal length in the vicinity of roots, N: number of tips on MMA, Nr: number of tips in the vicinity of roots, L/N: branch mean length on MMA, Lr/Nr: branch mean length in the vicinity of roots

A ratio >1.0 indicated that the treatment stimulated the parameter. Analysis of variances was then performed on these ratios. The results are reported in Table 2. Hyphal length was more than two times higher in the vicinity of fresh roots than on minimal media, whatever the pathogenicity of the strain. Branching of both strains Fo47 and Fol8 was more susceptible to the stimulation than the other strains, suggesting a better ability for these strains to exploit the root environment. However, the stimulations of the total hyphal length were not significantly different among the strains, indicating that the stimulation is not related to pathogenicity. It is worth noting that the L/N ratio was increased for all the strains in a comparable way. According to Trinci (1974) this clearly indicates that whatever the strain, their physiological state was modified in the root vicinity compared to what it was on minimal medium.

The mycelial development of strain Fo47, Fol8, and Forl22 in the vicinity of wheat and tomato roots were compared. For each of the three strains, no significant difference was found for any of the parameters when the strains were developing in the root vicinity of tomato or wheat. These results indicate that *F. oxysporum* is clearly a rhizosphere species. This species is susceptible to metabolites which are present in various rhizospheres and responsible for the stimulation of mycelial development.

To determine what was responsible for such a stimulation, actively exuded (called exudates) and passively diffusing (from freeze dried roots) root compounds, EtOH soluble and insoluble root components were tested. The results shown in Table 2 indicate that the highest stimulation factor was obtained with root exudates. However, comparable stimulations were

observed among the strains within a treatment and only few significant differences were noticed for one or an other of the three parameters measured. Therefore, the stimulating factors were likely to be small molecules, diffusing through the agar and acting similarly on the different strains. That is the reason why increasing concentrations of (i) glucose, (ii) casamino acids and (iii) glucose plus casamino acids were tested on the development of strain Fo47. Only the results obtained after 19 h are presented Fig. 2 (A-F) for sake of clarity. Whatever the glucose concentrations, there was no statistical difference within the hyphal length (Fig. 2A) and within the number of ramifications (Fig. 2B). Conversely, both parameters were significantly higher in the presence of EtOH extract. This indicates that the carbon source is not the stimulating factor for the extension and branching of the fungus.

Table 2. Mean ratios of a parameter (L, N, L/N) measured in a treatment (fresh roots, exudates, freeze dried roots, soluble and insoluble EtOH extracts) to the same parameter measured on MMA[z].

Parameter	strains	Fresh roots	Exudates	Freeze dried roots	soluble EtOH extracts	Insoluble EtOH extracts
Length (L)	Fo47	2.9 a	7.8 a	1.6 b	2.0 a	1.4 a
	Fol8	2.9 a	8.8 a	2.7 a	2.2 a	1.1 a
	Forl22	2.1 a	7.9 a	1.8 b	2.1 a	1.4 a
	Forl19	2.0 a	NT	NT	NT	NT
	Fol15	2.0 a	NT	NT	NT	NT
Number of tips (N)	Fo47	2.1 a	5.0 b	2.6 a	2.6 a	1.4 a
	Fol8	2.0 a	8.0 a	3.5 a	2.4 a	1.2 b
	Forl22	1.6 c	5.2 b	3.0 a	2.5 a	1.5 a
	Forl19	1.7 b	NT	NT	NT	NT
	Fol15	1.3 c	NT	NT	NT	NT
Branch mean length (L/N)	Fo47	1.4 a	1.6 a	0.6 b	0.8 a	1.0 a
	Fol8	1.5 a	1.1 a	0.8 a	0.9 a	1.0 a
	Forl22	1.3 a	1.7 a	0.6 b	0.8 a	1.0 a
	Forl19	1.2 a	NT	NT	NT	NT
	Fol15	1.5 a	NT	NT	NT	NT

[z] For each parameter, ratios followed by the same letters within a column are not significantly different ($P = 0.05$). NT = the strain has not been tested in this treatment.

When casamino acids were added to the medium, (Fig. 2C), the hyphal length significantly increased with a concentration of organic nitrogen of 0.125 mg/ml. For higher concentrations of casamino acids, the hyphal length remained constant. The value of the length at the plateau was significantly below the value of the hyphal length reached in presence of EtOH soluble extract A similar pattern was observed for the branching (Fig 2D). The hyphal length increased with increasing concentrations of glucose and casamino acids (Fig. 2E) but reached a plateau when concentrations of 0.25 and 1 mg/ml of respectively casamino acids and glucose were added to the MMA. The value at the plateau was not significantly different from the value in presence of EtOH. The branching (Fig. 2F) was still significantly higher in presence of EtOH soluble extract than it was in the other cases. This indicates that organic nitrogen is one of the main factors responsible for the stimulation of the extension and the ramification of the fungus.

Fusarium oxysporum is fundamentally a rhizosphere species. All the strains tested so far are strongly stimulated in the root vicinity due to the organic nitrogen containing root exudates. These compounds increase the conidial germination and induce metabolic changes resulting in higher mycelial elongation, higher branching and in some cases in a change in the size of the growth unit as defined by Trinci (1974). This higher development observed in the root vicinity compared to the one observed in the absence of root could result in turn in the dense network of mycelium observed in the vicinity of the root by Olivain and Alabouvette (1997). Although these experiments have been done in vitro using reduced systems and not in the rhizosphere per se, it is possible to suspect that such a dense network could allow *F. oxysporum* strains to colonize and utilize the root environment. Nevertheless, such a stimulation is not related to pathogenicity. It is neither fungal strain specific since the five strains were equally stimulated. Nor is it plant species specific since wheat and tomato had a similar effect.

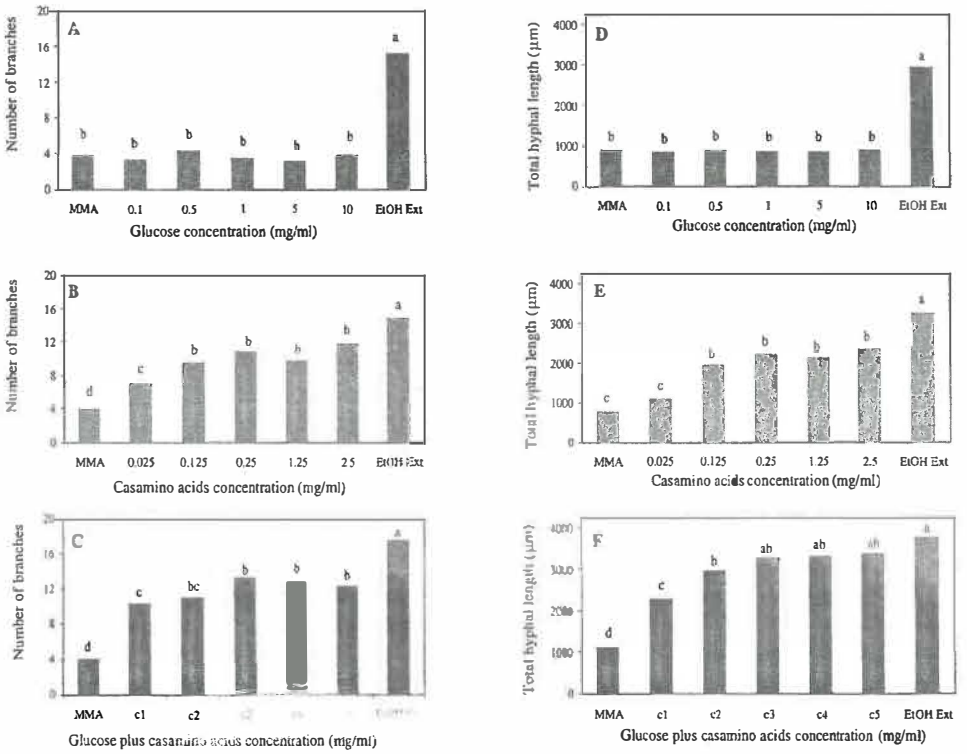

Fig. 2. Role of glucose (A, D), casamino acids (B,E) or glucose plus casamino acids (ratio 4:1) (C, F) on the number of tips (A,B,C) and the hyphal length (D,E,F) achieved by Fo47 when developping on an agar film. Bars with the same letters within a graph are not significantely different (P = 0.05) according to the Newman-Keuls test.

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Electrophoretic analysis of some *Trichoderma viridae* isolates and mutants

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Abstract

Among the modern methods of biochemical characterization of the control agents of plant pathogens, electrophoresis is frequently used (Popescu 1990, Sesan 1997). SDS-PAGE is a variant of zonal-type electrophoresis where migratation of SDS-denaturated proteins is almost exclusively dependent on molecular mass. This facillitates evaluation of protein fraction size using a suitable standard (Bailey 1967, Gordon 1974). The polyacrylamide support used in this method stabilizes the migration zones and allow separation of components by their physical and chemical parameters and the effective conditions in which electrophoresis is being performed (Iordachescu 1987, Jercan 1984).

Materials and Methods

Five wild-type isolates of *Trichoderma viride* Pers. ex S.F. Gray (Td 5, Td 35, Td 45, Td 49, Td 50) and 15 mutants resistant to the fungicides Tiradin 75 PU/TMTD (mutants Td A to Td K) and Topsin M 70/thiophanate methyl (mutants Td L to Td O) (Sesan 1985, Sesan 1986, Sesan and Baicu 1989) were used in this study (Table 1). All strains were tested for antagonistic activity against seven fungal species in dual culture as previously described (Jouan et al. 1964).

Table 1. Fungi used in this study.

Strain	Origin	Fungicide resistance
Trichoderma viridae wild-type		
Td5	Tomato roots	
Td35	Soybean seeds	
Td45	Bean seed	
Td49	Bean seed	
Td50	Bean seed	
Mutant derivatives of Td5		
TdA through TdK	(Sesan and Baicu 1989)	Tiradin
TdL through TdO	(Sesan and Baicu 1989)	Topsin
Alternaria alternata	Wheat kernels	
Botrytis cinerea Bc. 1	Strawberry fruit	
B.cinerea Bc. 2	Sunflower akenes	
Fusarium culmorum	Bean seeds	
F. graminearum	Maize kernels	
Pythium ultimum	Sugarbeet seedlings	
Sclerotinia sclerotiorum	Carrot roots	
Stemphylium radicinum	Carrot roots	

SDS-PAGE analysis was used to reveal protein bands and to determine protein molecular mass. A Mini-PROTEAN II (BIO-RAD) was used in variant with vertical plates following the Laemmli procedure. Reagents and solutions used where as follows: (i) Acrylamide-bisacrylamide system (14.6 g acrylamide, 0.4 g bisacrylamide, 50 ml distilled water); mixture filtered and kept at 40 °C; (ii) TRIS-HCl buffer pH 8.8, 1.5M (27.23 g basic TRIS, 80 ml distilled water), pH adjusted with distilled water in a volume of 150 ml; kept at 40 °C; (iii) TRIS-HCl buffer pH 6.8, 0.5M (6 g basic TRIS, 60 ml distilled water), pH adjusted with HCl 1N and brought to a volume of 100 ml with distilled watter; (iv) SDS solution 10% (10 g SDS, 100 ml distilled water); (v) Denaturant buffer for samples (4 ml distilled water, 1 ml TRIS-HCl buffer, pH 6.8, 0.8 ml glycerol, 1.6 ml SDS 10%, 0.4 ml 2-mercaptoethanol, 0.2 ml bromphenol blue 0.05%); (vi) Migration buffer, pH 8.3 (14.4 g glycerine were dissolved in 500 ml distilled water and pH adjusted to 7.8-7.9 with NaOH 1N. 3.025 g basic TRIS have been added and volume brought to 1,000 ml with distilled water); (vii) Staining solution Comassie Brillant Blue (0.1 g Comassie Brillant Blue R-250, 40 ml methanol, 10 ml glacial acetic acid, 100 ml distilled water); (viii) Destaining solution (40 ml ethanol, 10 ml acetic acid, 100 ml distilled water); (ix) Migration gel solution (4.1 ml distilled water, 2.5 ml TRIS-HCl pH 8.8, 100 µl SDS 10%, 3.25 ml acrylamide-bisacrylamide, 50 µl ammonium persulphate 10%, 20 µl TEMED; (x) Concentrating gel solution 4% (6.1 ml distilled water, 2.5 ml TRIS-HCl, pH 6.8; 100 µl SDS 10%; 1.3 ml acrylamide-bisacrylamide, 50 µl ammonium persulphate 10%; 20 µl TEMED; (xi) Drying solution (44 ml methanol; 44 ml distilled water; 2 ml glycerine).

Analysis performance included the following steps: (i) Preparation of migration vertical plates by pouring the concentration gel, cooling the gel, pouring the migration gel, practicing cavities in gel by means of a matrix; (ii) Denaturation of samples by diluting 1 : 1 with denaturating buffer 5 and heating to 95 °C for 5 min; (iii) Migration conditions: after denaturating samples in gel cavities of migration buffer 6 in the equipment, migration was achieved for 45 min, at a tension of 200 V; (iv) Gel staining was done by placing it in the staining solution Comassie Brillant Blue for 1 h; (v) Gel destaining after staining was obtained by its transfer to the destaining solutionand keeping it there until the gel became translucid, while the protein bands turned to bright blue color; (vi) Gel drying was obtained by placing it after destaining in the drying solution.

Analysis procedure for protein bands after gel drying consisted of measurement of migration distances with a ruler (distance between the start point and the point of apparition of protein bands) both in samples and standard. The standard used to determine the molecular mass of proteins specific to every sample was SERVA-type and contained: phosporylase B, BSA, ovalbumine, carbonic anhydrase.

Results and Discussion

The retention factor and molecular mass of standard proteins are shown in Table 2 and Fig. 1. Graphic display of relation between the retention factor and the logarithm of molecular mass is shown in Fig. 2. Biochemical characterization of the wild-type *T. viride* isolates (Fig. 1, Table 3) indicated that (i) proteins with molecular masses 7, 8, 10, 12, 14, 35, 56, 60 kD represent characteristics for genus and species that are easily differentiated; (ii) within the same species the quantitative differences appeared at the level of proteins with molecular masses of 29, 45, 87, 89 kD and are determined by provenance of various isolates belonging to the same species; (iii) isolates Td 49 and Td 50 exhibited strong similarities having a

common provenance. Both Td 49 and Td 50, but especially Td 50, had high antagonistic activity against all seven pathogens tested (Table 4).

Table 2. The retention factor (*Rf*) and molecular mass of standard proteins

Standard protein	Retention factor (*Rf*)	Molecular mass (kD)
B Phosphorylase	0.15	97.4
B S A	0.27	67.0
Ovalbumine	0.44	45.0
Carbonic anhydrase	0.55	29.0

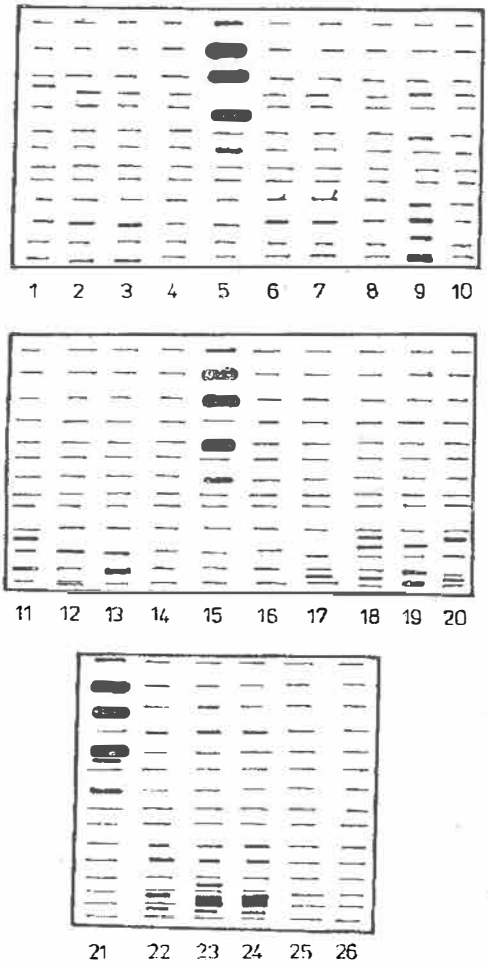

Fig. 1. Protein bands from SDS-PAGE analysis of *Trichoderma* wild-type and mutants compared to standard proteins. Lanes 1 to 4, 6 to 14, 16 to 17, and 22 to 24 correspond with fungicide resistant mutants TdA to TdO. Lanes 18 to 20 correspond with wild-type Td Norvegia, Td5, Td35. Lanes 22 to 24 correspond with wild-type Td45, Td49, Td50. Lanes 5, 15, and 21 correspond with standards.

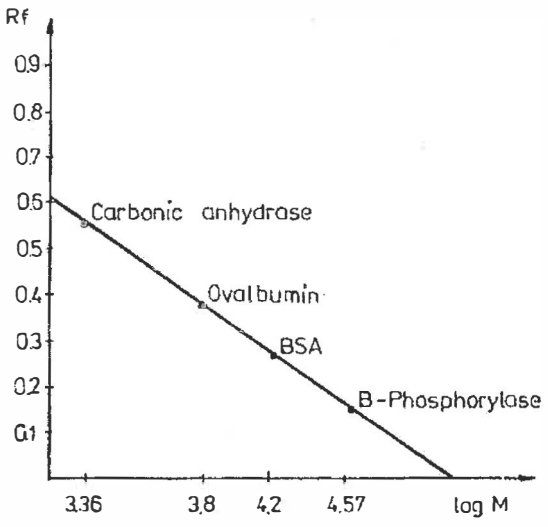

Fig. 2. Relation between the retention factor (Rf) and the logarithm of molecular mass (log M).

Table 3. Biochemical characterization of some *Trichoderma viride* isolates and mutants based on protein bands separated[z]

M/kD	7	8	10	12	14	29	35	45	56	60	89	97
Rf	0.98	0.93	0.89	0.84	0.78	0.55	0.49	0.44	0.33	0.31	0.18	0.15
Td A	+	+	+	++	+	-	±	-	+	++	-	-
Td B	++	+	+	++	+	-	±	-	±	++	-	-
Td C	++	+	+	++	+	-	+	-	+	+	-	-
Td D	-	-	±	+	-	-	-	-	-	+	-	-
Td E	+	+	+	++	+	-	±	-	+	++	-	-
Td F	++	+	+	++	+	-	±	-	+	++	-	-
Td G	+	+	+	+	±	-	±	-	-	+	-	-
Td H	++	++	++	++	++	-	++	-	+	++	-	-
Td I	++	++	+	++	+	-	±	-	±	+	-	-
Td J	++	+	++	++	++	-	+	-	+	++	-	-
Td K	++	+	+	++	±	-	±	-	+	++	-	-
Td L	++	+	++	++	++	-	+	-	++	++	-	-
Td M	++	+	++	+	++	-	+	-	+	++	-	-
Td N	++	+	++	++	±	-	±	-	+	+	-	-
Td O	+	++	++	+	+	+	+	+	+	++	±	±
Td 5 (control)	+	++	++	++	++	-	+	-	+	+	-	-
Td 35	++	+	+	++	++	-	++	-	+	+	-	-
Td 45	++	++	±	++	++	±	±	±	+	±	±	±
Td 49	++	++	++	++	++	±	++	+	+	++	++	++
Td 50	++	++	++	++	++	±	++	+	+	++	±	±

[z]Band intensity: '-' no band, '±' weak band, '+' intense band, '++' very intense band.

Biochemical analysis of *T. viride* mutants obtained from the isolate Td 5, revealed that proteins with molecular masses of 7, 8, 10, 12, or 14 kD were found in all isolates and mutants (Fig. 2). This may be partly responsible for fungicides resistance in these mutants. Proteins with molecular masses of 35 or 56 kD were found in higher amounts compared with the wild-type Td 5. These proteins could represent resistance inhibitors to certain fungicides. Lower amounts of 35kD and 56 kD proteins in *T. viride* mutants confers resistance to the fungicides Tiradin 75 PU and Topsin M 70. A 60 kD protein was present in generally higher amount in mutants compared to the initial isolate Td 5. This may contribute to increased antagonistic activity of mutants compared to wild-type Td 5 (Table 4). The fifteen *T. viride* fungicide resistant mutants have been field-tested for protection of grapevine against grey mould caused by *Botrytis cinerea* (Sesan and Podosu 1993, Sesan and Teodorescu 1993, Sesan et al. 1995). The most active were Td E, Td M, Td K and Td N. The strong biochemical activity could be induced by the presence of high amounts of 35 and 56 kD proteins.

Table 4. Antagonistic activity of *Trichoderma viride* isolates and fungicide resistant mutants[z].

Tricho-derma	*Fusarium gramine-arum*	*Fusarium culmorum*	*Pythium ultimum*	*Botrytis cinerea* Bc1	*Botrytis cinerea* Bc2	*Alternaria alternata*	*Sclerotinia sclerotio-rum*	*Stemphylium radicinum*
Td 35	0.25	0.40	0.24	0.44	0.42	0.40	0.22	0.35
Td 45	0.78	0.86	0.72	0.70	0.80	0.86	0.48	0.52
Td 49	0.28	0.38	0.48	0.62	0.58	0.90	0.76	0.90
Td 50	0.30	0.38	0.36	0.35	0.38	0.42	0.54	0.75
Td 5 (control)	0.55	0.39	0.54	0.89	0.92	0.54	0.45	0.30
Td A	0.42*	0.28**	0.30***	0.30***	0.29***	0.49*	0.45	0.26
Td B	0.31***	0.35	0.43**	0.36***	0.32***	0.50	0.43	0.27
Td C	0.32***	0.29**	0.30***	0.53***	0.34***	0.44***	0.44	0.26
Td D	0.39**	0.31**	0.37***	0.17***	0.39***	0.32***	0.42	0.25
Td E	0.23***	0.24***	0.34***	0.23***	0.39***	0.42***	0.42	0.25
Td F	0.29***	0.31**	0.32***	0.33***	0.34***	0.56	0.41	0.26
Td G	0.46	0.28**	0.36***	0.37***	0.33***	0.44***	0.39	0.26
Td H	0.35***	0.27***	0.36***	0.21***	0.50***	0.52	0.41	0.27
Td I	0.35***	0.31**	0.20***	0.23***	0.40***	0.35***	0.40	0.26
Td J	0.43*	0.23***	0.22***	0.41***	0.34***	0.49*	0.39	0.27
Td K	0.44*	0.27***	0.29***	0.19***	0.31***	0.36***	0.38	0.26
Td L	0.39**	0.41	0.23***	0.39***	0.45***	0.50	0.42	0.26
Td M	0.27***	0.31**	0.26***	0.36***	0.33***	0.39***	0.36	0.26
Td N	0.36***	0.26***	0.23***	0.26***	0.35***	0.40***	0.38	0.25
Td O	0.25***	0.25***	0.39***	0.23***	0.37***	0.33***	0.44	0.26

[z] Antagonistic activity was evaluated based on based on **x** coefficient following Jouan et al. (1964). Significant differences were observed for mutants compared with the wild-type Td5 control at $P < 0.05$*, $P < 0.01$** and $P < 0.1$***.

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Capacity of a bacterial inoculant to degrade the alkaloids produced by the roots of bindweeds: does it contribute to rhizosphere colonisation?

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Abstract

The roots of certain plant species, especially the morning glories *Calystegia sepium* (hedge bindweed) and *Convolvulus arvensis* (field bindweed) synthesise calystegines, a group of secondary metabolites belonging to the family of tropane alkaloids. The capacity to degrade calystegines is uncommon in rhizosphere bacteria and these alkaloids are thought to act as nutritional mediators of plant-microbe interactions by favouring calystegine-degrading microorganisms in the rhizosphere. In *Rhizobium meliloti* 41, the genes *cac* responsible for calystegine catabolism are located on pRme41a, a 225-kb self-transmissible plasmid not implicated in the symbiosis with *Medicago sativa* roots. The strain can use these alkaloids as sources of carbon and nitrogen. It might be useful to introduce the ability to degrade calystegines into bacterial strains selected for their capacity to affect bindweed development. Such modified strains might exhibit enhanced biocontrol activity if they can colonise the rhizosphere of bindweeds at higher population levels. The selective advantage due to the *cac* genes in the rhizosphere of calystegine producers was evaluated in soil microcosm experiments using *R. meliloti* 41, a derivative of 41 cured of the plasmid pRme41a, and a complemented strain with restored calystegine-degradation ability. The presence of the *cac* plasmid pRme41a was not required for colonisation of the rhizosphere of calystegine-producing plants. However, competition experiments showed that the plasmid contributed to the competitiveness of rhizobia for colonisation of the rhizosphere of calystegine-producing plants but not of calystegine-negative plants. This finding raises the possibility that biocontrol strains with enhanced ecological fitness in the bindweed rhizosphere may be developed by introducing heterologous *cac* genes into promising wild-type strains.

A PCR/RFLP analysis of 16S rDNA from *Pseudomonas* spp. suppressing fungal diseases in cereals

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Abstract

Bacterial isolates tested for biocontrol effects against fungal diseases in cereals were grouped using RFLP patterns. The 16S rDNA region from each of 38 fluorescent pseudomonad root isolates and from eleven *Pseudomonas chlororaphis* reference strains was PCR amplified and digested with restriction endonucleases. The reference strains were chosen since in biochemical tests the root isolates showed the closest relation to the *P. chlororaphis* species.

On the basis of RFLP patterns from three restriction endonucleases, *Alu*I, *Msp*I and *Rsa*I, a group of ten root isolates, showing the same pattern, was descriminated from the other root isolates and from the reference strains. Each of these ten isolates effectively suppressed fungal diseases in cereals in greenhouse tests. The *Alu*I RFLP pattern of the ten isolates was different from that of eight *P. chlororaphis* reference strains (USDA B1854, USDA B1869, USDA B2075, USDA B14869, USDA B14874, NCTC 10686, DSM 6508 and ATCC 17811). With a combination of the restriction endonucleases *Msp*I and *Rsa*I the other three *P. chlororaphis* reference strains (NCTC 7375, ATCC 9446 and ATCC 17414) could be discriminated.

Risk assessment and monitoring

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Risk assessment associated with the release of a genetically modified *Pseudomonas fluorescens* in the field

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Abstract

The first release into the environment of a genetically modified free-living (non-symbiotic) bacterium in the UK was made in 1993. The genetically modified microorganism (GMM) was *Pseudomonas fluorescens* SBW 25, marked on the chromosome in two well-separated, presumed non-essential sites, with the *lacZY* marker gene cassette that provides ability to utilize lactose and another gene cassette comprising the kanamycin resistance gene (*aph*) and the *xylE* gene that confers ability to degrade catechol. The releases were made by seed and spray applications onto wheat in a silty loam soil at Littlehampton and by seed application only onto sugar beet in a heavy clay soil at Oxford. Survival, establishment, dissemination, gene transfer and impact of the GMM on indigenous microbial populations was examined. Effects on impact were compared with those of the unmodified wild-type. In the field, the GMM could colonize wheat and sugar beet plants from a seed inoculation and survived on decaying wheat roots for up to 300 days after harvest at levels of 10^5 CFU/g root tissue. Three years after the release the GMM was no longer detectable in soil. With wheat, the GMM was able to spread vertically down to a depth of 45 cm and laterally up to 2 m but no dissemination was found from sugar beet. No transfer of the chromosomal markers from the GMM to the indigenous *Pseudomonas* population was found although transfer of mercury resistance plasmids from the indigenous microbial population to the GMM was detected at the Oxford site. The release of the GMM and wild-type resulted in significant, but transient, perturbations of some components of the microbial community of wheat. Nevertheless, the effects of the wild-type and GMM were not significantly different. The implications of this work for future releases of GMMs are discussed.

Introduction

The techniques for genetic modification of both bacteria and fungi are now well advanced and have been used to modify activity of several biological disease control agents. For long term control, such genetically modified microorganisms (GMMs) should establish and survive in the target ecological niche. However, their use is not without risk to the environment and, before widespread use is possible, evaluation of both the benefits and possible harm to man and the environment is needed. As an initial phase of the risk assessment process, a study was undertaken to increase the understanding of the behavior of free-living GMMs in the environment by inserting genes that enable detection, selection and quantification of the GMM on simple culture media. The insertion of two marker gene cassettes into *Pseudomonas fluorescens* SBW 25 enabled sensitive detection of cells in the environment and also allowed the possibility of detecting gene transfer of the inserted genes to related organisms. This GMM was then used in a series of contained glasshouse experiments involving wheat grown in a silty loam soil at Littlehampton and sugar beet grown in a heavy clay soil at Oxford (De

Leij et al. 1994a, b, Ellis et al. 1995, Thompson et al., 1995a). Based on information from these pre-release studies, permission for the field release was given by the Secretary of State for the Environment in 1993. Details of these releases have been published elsewhere (De Leij et al. 1995a, b, Thompson et al. 1995b, Lilley et al. 1997a, b) but the key results are listed here together with the recently completed post-release monitoring data. The implications of this work are discussed.

Materials and Methods

Genetic modification. A plasmid-free *P. fluorescens* SBW 25 from the phylloplane of sugar beet was chosen for modification as this species was commonly found on the phylloplane and rhizosphere of wheat and sugar beet, was considered non-pathogenic to humans, animals and plants, and was not antagonistic to other microbial inhabitants of the phytosphere. The first marker gene cassette contained the *lacZY* genes which confered ability to utilise lactose and also allowed detection in media containing the chromogenic substrate (X-gal) to yield a blue product. The second marker gene cassette, *aph-xylE*, encoded for resistance to the antibiotic kanamycin (*aph*) and for the ability to degrade catechol *(xylE)* to yield a yellow product. The marker genes were introduced 1M bp apart (15% of the genome) on the chromosome (Bailey et al. 1995, Rainey and Bailey 1996). It was the first time a doubly marked GMM had been used in risk assessment studies. The GMM exhibited similar behaviour to the non-modified wild-type and the marker genes allowed sensitive detection from environmental samples [1 cell/10 g soil using a most probable number broth enrichment method and 20 CFU/g by direct planting on selective agar (De Leij et al. 1993)] and assessment of gene transfer (Lilley et al. 1996).

Field release - wheat. The site at Littlehampton, West Sussex on a silt loam soil was situated in the middle of a 3 ha spring wheat field with a cropping history of wheat for the previous four years. Fifteen plots of five blocks each of untreated, wild-type inoculated and GMM inoculated were established in April 1993. Wheat seeds (cv. Axona) were inoculated by vacuum infiltration and fluid drilled. Each 1.5 x 1.5 m block received approximately 2,000 seeds with 10^7 to 10^8 CFU/seed. At tillering, the wild-type and GMM blocks were sprayed with 100 ml of bacterial suspension containing 3.2×10^{10} CFU/ml of the wild-type and genetically modified *P. fluorescens*, respectively. The crop was harvested 20 weeks after planting and the plots left fallow until April 1994 when half of each block was replanted with wheat. After one month, wheat seedlings were harvested and assayed for colonization by the GMM. The plants were then kept fallow by application of herbicides until December 1996 when the experiment was terminated. Throughout the release experiment, wheat plants, weeds and soils were collected and assayed for colonization by the GMM. Impact of the released bacteria on indigenous microbial populations on wheat and transfer of marker genes from the GMM to the indigenous microbial populations were also assessed.

Field release - sugar beet. The GMM was applied as a seed dressing (7×10^6 CFU/seed) to commercially pelleted sugar beet (*Beta vulgaris* cv. Amethyst) in April 1993 and planted in 10 x 10 arrays surrounded by three rows of untreated plants in clay soil at the University farm, Wytham, Oxfordshire. Nine individual 5 m_2 plots of sugar beet were established, three each of untreated, wild-type and GMM treated. The survival and persistence of the GMM, together with its dispersal, were monitored until the crop was harvested in January in 1994. A number of plants were left in each plot so that persistence of the GMM on over-wintered sugar beet could be determined. In April 1994 and 1995, untreated sugar beet seeds were sown in half

the area of each of the plots and monitored for colonization by the GMM. Throughout the release, sugar beet plants, weeds and soil were collected and assayed for colonization by the GMM. Periodically, insects were also collected and assessed for the presence of the GMM. Impact of the released bacteria on indigenous microbial populations on sugar beet and transfer of marker genes from the GMM to the indigenous microbial populations were also assessed. Mercury resistance was used to evaluate gene transfer from indigenous microbial populations to the GMM.

Results

Survival, establishment and dissemination. The GMM survived and established in the rhizosphere of wheat at levels of approximately 10^6 CFU/g root tissue and survived on decaying wheat roots up to 300 days after harvest at levels in excess of 10^5 CFU/g root tissue. The variation between samples and sampling occasions was small.

When the GMM was applied onto wheat seeds, only the first emerging wheat leaves became colonized. But, when applied as a spray application to young plants, the GMM multiplied and established in large numbers (>10^5 CFU/g tissue) on the leaves of wheat, but not the ear.

Although the GMM was able to colonize the rhizosphere as well as the phylloplane of sugar beet, the levels of colonization and subsequent establishment varied dramatically between plants and sampling occasions. For example, GMM numbers at the root surface varied over the season between 1 and 10^6 CFU/g tissue.

The GMM was able to disseminate vertically up to a depth of 45 cm and laterally up to 2 m away from the point of inoculation in silty loam soil, considerably further than similar studies carried out in the USA and Australia. No dissemination was found in heavy clay soil when GMMs were applied to the clay seed coating of sugar beet seeds.

Numbers of GMMs declined steadily in non-rhizosphere soil and were not detected in soil at Littlehampton after June 1994 or at Oxford after October 1994.

No insects tested in this study were found to disseminate GMMs.

Although, the GMM could be transferred from inoculated to non-inoculated plants by foliar contact, dissemination did not extend beyond one row of non-inoculated plants.

Despite precautions to minimize spray drift, extensive aerial dissemination of GMMs to the surrounding environment occurred when GMMs were sprayed onto young wheat plants. However, subsequent establishment of these GMMs was limited.

Young plant tissue had an enriching effect on GMM populations. The GMM re-established in the rhizosphere and phylloplane of volunteer wheat, resown wheat and in the phylloplane of resown sugar beet even though the GMM was often non-, or barely detectable, in soil. Similarly, GMMs were present on newly developing sugar beet leaves and on over-wintered plants. They were absent from old leaves that had over-wintered.

Residual wheat root tissue acted as a reservoir for GMMs in the soil.

Dissemination and establishment of GMMs on weeds were highly variable. From more than 100 samples taken during the first year following release, only roots of scarlet pimpernel (*Anagallis arvensis)* and leaves of buttercup (*Ranunculus* sp.) were consistently colonized (>10^3 CFU/g tissue). The GMM was last detected at Oxford on three weed species and replanted sugar beet in October 1994 and at Littlehampton in a single root sample from a grass (*Holcus* sp.) in June 1995.

Different results were found in the field from those expected using glasshouse microcosm studies. For example, GMM survival on wheat leaves after spraying, and on wheat roots after harvest, was greater than expected. In contrast, relative colonization of sugar beet leaves and weed species by the GMM was much less than expected.

Gene transfer. No evidence was found for the transfer of the chromosomal markers (*lacZY* and *aph-xylE)* from the GMM to indigenous *Pseudomonas* populations after the release of the GMM into the phytosphere of wheat and sugar beet.

Conjugative plasmid transfer of mercury resistance from indigenous microbial populations to the GMM, was estimated to occur at rates in excess of one transconjugant per 3×10^7 potential recipients. Thus, the probability of gene transfer to GMM inocula may be greater than previously predicted.

Impact. The release of wild-type and genetically modified *P. fluorescens* resulted in significant, but transient perturbations of some components of the microbial community in the phytosphere of wheat. But no significant difference was detected between the microbial communities on plants that were inoculated with GMMs compared with those inoculated with the non-modified *P. fluorescens*.

Microbial communities in soil were, in general, not affected by the release of either wild-type or genetically modified *P. fluorescens*.

Estimations of some specific microbial populations, especially yeasts, were sensitive enough to detect microbial perturbations caused by releases of microbial inocula in the phytosphere.

The observed microbial perturbations that resulted from the release of GMMs were of no environmental significance because the effects of wild-type and GMM were not significantly different, effects were transient, and perturbations had no noticeable effect on plant health and growth.

Discussion

The results on survival, establishment and dissemination showed that the genetically modified *P. fluorescens* used in this release study was more persistent, and its dissemination on the inoculated plant was greater, than could be predicted from the pre-release experiments that were conducted under contained conditions in microcosms and pot experiments. The survival and establishment was also greater than that found by other independent release studies with GMMs. Consequently, while pre-release experiments in contained environments are thought to be necessary before any GMM is released into the environment, their value is somewhat questionable since they might underestimate the environmental risks associated with GMM survival and establishment. Nevertheless, since this work was carried out with a non-pathogenic bacterial strain that was modified purely for monitoring purposes, this under-estimation of GMM survival and establishment did not result in the occurrence of any hazard or risk to the environment.

Differences in survival, establishment and dissemination of the GMM found in different host plants, weeds and soils suggests that these variables should be considered in any environmental risk assessment.

The environmental risks associated with the release of GMMs that are modified with genes for monitoring purposes only, are probably the same or less than the risks associated with the releases of non-modified microorganisms because of the extra genetic load carried by the GMM. The insertion of marker genes does not necessarily add to the environmental risk,

especially if the phenotypic characteristics of the marker genes are already expressed in the release environment. The use of such GMMs in scientific studies should not be restricted because of the inserted genetic elements.

No evidence was found that the inserted genes were mobilized. This was probably due to their insertion on the chromosome and the lack of selection pressures which would benefit possible recipient organisms. The observation that plasmid transfer occurred in the phytosphere from indigenous microbial populations to the GMM more frequently than predicted, suggests that better methodology should be developed to investigate the frequency and consequence of gene transfer for risk assessment purposes. To minimize the potential for gene transfer, introduced gene sequences should be located on the chromosome of the parental strain.

Pre-release studies with GMMs in microcosms under containment should aim to test the consequences of a release in experimental systems that mimic realistic situations of use. Although the soil/rhizosphere environment can be simulated to some extent in the glasshouse, the phylloplane environment is more difficult to mimic and use of controlled environment facilities would help in this work.

Pre-release studies should also examine "worst case scenarios", where optimal conditions are provided for the GMM and its effects are tested in situations where a release is predicted to result in environmental harm. In addition, unrealistically large doses of GMMs could be tested in such pre-release studies.

Environmental risk assessments that are based on GMM impact on indigenous microbial populations are difficult to interpret because of the lack of knowledge on activity and ecosystem function of microbial populations in the environment. Therefore, estimates of microbial perturbations that result from the release of a GMM could be complemented by estimates of functional perturbations, such as effects on plant growth and plant health, nutrient cycling, nutrient uptake by the plant and effects on soil characteristics. Such functional effects can be interpreted and used in an environmental risk assessment.

If GMMs modified for the improved control of pests and diseases or environmental remediation have a fitness advantage in the target environment, they should be less fit than the non-modified wild-type in the non-target environment, to avoid possible unwanted establishment in the non-target environment.

The release of any substance into the environment, whether it is a chemical or micro-organism, should be evaluated for both the benefits and possible harm to man and the environment. Excessive legislation for the use of GMMs might, in fact, lead to more environmental harm if GMMs that could provide environmentally safe solutions for the control of pests and diseases or environmental remediation are delayed in their development.

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Molecular approaches in biocontrol of Fusarium wilt of carnation

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Abstract

Different strains of *Fusarium oxysporum* antagonistic to phytopathogenic formae speciales of *F. oxysporum* were transformed by insertion of the *benA* or *hph* genes. The frequency of transformation ranged from about 3-8 to 800-1000 transformants per microgram of DNA. Plasmids pbenA33 and pAN7-1 usually integrated in the genome, but most of the pAN7-1 transformants tested underwent loss of plasmid copies during in vitro growth without selective pressure or after release in soil microcosms, by retaining the hygromycin B resistance character. On the contrary, the plasmid vector pLD did not integrate in the chromosomes of *F. oxysporum* and transformants lost the resistant phenotype on non-selective media. Biocontrol experiments against *F. oxysporum* f.sp. *dianthi* revealed that genetically manipulated strains can perform very differently as biocontrol agents. Results are discussed in relation to risk assessment of the release of transgenic antagonists and to the possible identification of tagged mutations in the biocontrol potential of this fungus.

Introduction

Saprophytic *Fusarium* spp., isolated from *Fusarium*-suppressive soils, are highly effective in reducing vascular wilts caused by formae speciales of *F. oxysporum* (Alabouvette 1986, Garibaldi et al. 1986, 1990, 1994, Lemanceau and Alabouvette 1991, Minuto et al. 1995, Tramier et al. 1983). Several genetic transformation protocols were developed for pathogenic *Fusarium* spp., some of which have been used in monitoring antagonistic strains of *F. oxysporum* with resistance markers (Gullino et al. 1995). Integrative transformation with non-homologous plasmids may also be used to elucidate the molecular basis of biocontrol in antagonistic *Fusarium* spp. strains by generating tagged mutations. The aim of this work was to evaluate the potential of genetic transformation for strain improvement in antagonistic *F. oxysporum*. Eighteen transformants, obtained by the insertion of plasmids pbenA33 and pAN7-1, were applied in biocontrol experiments against *F. oxysporum* f.sp. *dianthi* on carnation in order to evaluate if random integration of foreign DNA sequences may have modified the biocontrol potential of the recipient strains. In addition, both hygromycin B resistance and mitotic stability of pAN7-1 and pLD transformants were evaluated after in vitro growth in the absence of hygromycin B selective pressure and after release of the transformants in soil microcosms.

Materials and Methods

The following antagonistic *Fusarium oxysporum* strains were used in transformation experiments: 141 WT, 251 WT and 233 WT, isolated from the rhizosphere of carnation grown in a *Fusarium*-suppressive soil in Italy and sensitive to benomyl and to hygromycin B. The dark red-pigmented mutant 233/1 C5 is able to grow in the presence of >30 μg/ml benomyl,

but is sensitive to hygromycin B. This mutant was selected for altered pigmentation from the benomyl resistant (MIC >30 µg/ml) mutant 233/1, which was obtained by a 30-min UV treatment from isolate 233 WT. The nine transformants coded T8.1, T8.2, T8.3, T8.4, T8.5, T8.6, T8.7, T8.8 and T8.9, derived from isolate 141 WT, are able to grow in the presence of >30 µg/ml benomyl.

The transformants T26.1, T26.6, T26.19, T26.40, T27.7, T27.11, T27.28, T28.9, and T28.26 all derived from mutant 233/1 C5, are able to grow in the presence of >100 µg/ml hygromycin B and >30 µg/ml benomyl. A virulent strain of *F. oxysporum* f.sp. *dianthi* (pathotype 2), originally isolated from a wilted carnation plant grown in Liguria, Northern Italy and coded as F75, was used throughout the biocontrol experiments.

Protoplast isolation and transformation were carried out by the method of Langin et al. (1990). The following plasmids were used in the experiments: pbenA33, which carries a β-tubulin gene (*ben*A) of *Aspergillus nidulans* (May et al. 1987), pAN7-1 (Punt et al. 1987), which carries the hygromycin B phosphotransferase gene (*hph*) of *Escherichia coli*, and pLD (Kistler and Benny 1992), which contains the *hph* gene and a terminal, tandemly repeated hexanucleotide telomeric sequence $(TTAGGG)_{18}$ of *F. oxysporum*. In all experiments, parallel transformation controls were included in which the vector was omitted from the transforming mixture.

DNA isolated as described (Migheli et al. 1996) from nine pAN7-1 transformants grown in vitro under selective pressure or after four serial transfers on non-selective medium, and reisolated after 4 weeks from inoculation in carnation rhizosphere were digested with the restriction enzyme *Eco*RI which cuts twice in pAN7-1. Digested DNAs were subjected to agarose gel electrophoresis and transferred onto Hybond N^+ nylon membranes by following standard methods (Sambrook et al. 1989). Probe labelling, hybridization and chemiluminescent detection of the hybridizing bands with Lumigen PPD (Boehringer) were all performed according to manufacturer recommendation.

Six biological control experiments against *F. oxysporum* f.sp. *dianthi* on carnation were carried out under contained conditions as described previously (Migheli et al. 1996) by using nine pbenA33 and nine pAN7-1 transformants.

Results and Discussion

In twenty independent experiments, PEG-mediated transformation of antagonistic *F. oxysporum* was accomplished with a maximum efficiency of 3.2 and 8.4 transformants per µg of plasmid DNA for plasmids pbenA33 and pAN7-1, respectively. Transformation efficiency was considerably higher (up to 1,000 transformants per µg of DNA) for plasmid pLD (Migheli et al. 1995, 1996). When undigested DNA from the transformants was hybridized with the vector DNA, hybridization occurred only with high molecular weight DNA. This indicates that the transforming plasmids pbenA33 and pAN7-1 had integrated into the chromosomes (data not shown). In contrast, plasmid pLD did not integrate in the chromosomes of *F. oxysporum*.

During four rounds of in vitro growth in the absence of hygromycin B or after 4 weeks from release in soil microcosms, eight of nine pAN7-1 transformants showed stable inheritance of the hygromycin B resistance phenotype. As expected, pLD transformants were no more able to grow in the presence of hygromycin B after one or two rounds of cultivation on non-selective medium. Southern analysis revealed that only one pAN7-1 transformant underwent no obvious DNA rearrangement, while the other transformants showed different

degrees of rearrangement of the transforming DNA, mostly consisting of loss of plasmid copies (Migheli et al. 1996). Large-scale release of genetically manipulated organisms requires careful evaluation of their behavior in natural and agricultural environments (Cairns and Orvos 1992, Day and Fry 1992, Gullino et al. 1995). The genetic stability of transformed biocontrol fungi is crucial in evaluating the environmental risk of their release. Genetic instability in contained preliminary experiments may lead to erroneous conclusion in risk evaluation (Leslie and Dickman 1991). The high level of mitotic instability observed in our studies is not surprising in the case of multicopy transformants, where homologous recombination events between plasmid copies scattered around the genome might be responsible for the observed rearrangement of transforming DNA. Thus, alternatives should be investigated in order to increase the level of stability in this fungus, such as the identification of trapping DNA sequences in the *Fusarium* genome and their use in constructing new plasmid vectors, restriction enzyme-mediated integration (REMI, Schliestl and Petes 1991) of plasmid DNA, and the use of gene tagging and transformation systems based on the *Fot1* and *Impala* transposable elements, recently discovered in *F. oxysporum* (Daboussi 1996).

Table 1. Effect of different antagonistic strains of *Fusarium oxysporum* (nine benomyl resistant 141 WT-derived transformants and their recipient strain) on Fusarium wilt of carnation (cv. Indios)[z]

	Disease index (0-100)		
Strain	Experiment 1	Experiment 2	Experiment 3
none	45.0 b	70.0 d	82.5 c
141 WT	10.0 a	7.5 ab	10.0 a
T8.1	2.5 a	0 a	20.0 ab
T8.2	5.0 a	20.0 c	37.5 b
T8.3	10.0 a	5.0 ab	20.0 ab
T8.4	2.5 a	5.0 ab	17.5 ab
T8.5	7.5 a	0 a	35.0 b
T8.6	5.0 a	5.0 ab	12.5 a
T8.7	12.5 a	7.5 ab	27.5 ab
T8.8	2.5 a	10.0 a-c	32.5 b
T8.9	2.5 a	15.0 bc	40.0 b

[z] Values in a column followed by the same letter are not significantly different at $P = 0.05$ based on Tukey's multiple comparison test.

Most of the nine pbenA33 transformants tested in the first series of experiments were able to significantly contain the pathogen. The biocontrol efficacy was particularly evident in the first two trials where disease index was reduced from 45-70 to 0-20. In the third trial, the transformants were less effective, probably due to the high incidence of Fusarium wilt (Table 1). In the second series of experiments, the wild type reference strain 233 WT, the mutant 233/1 and the recipient strain 233/1 C5 effectively reduced the incidence of disease from 65-84 to 0-24. Among the nine transformants tested, only T26.40 consistently reduced the severity of Fusarium wilt giving a disease index rating of 9 to 20 (Table 2).

Our results show that genetically manipulated strains originating from the same transformation experiment, and deriving from the same recipient strain, can perform

differently as biocontrol agents against Fusarium wilt of carnation. Such a high variability in the antagonistic potential against *F. oxysporum* f. sp. *dianthi* was observed between fusants of *F. oxysporum* deriving from the same inter-strain cross (Migheli et al. 1992), and even between monoconidial isolates of the same benomyl resistant mutant. None of the transformants, however, had completely lost its antagonistic potential against Fusarium wilt of carnation. These results, and the fact that all the tested strains were characterized by multicopy integration of pAN7-1 and pbenA33, make these two series of transformants unsuitable for the identification of tagged mutations in biocontrol controlling genes.

Table 2. Effect of different antagonistic strains of *Fusarium oxysporum* (nine hygromycin B resistant transformants deriving from mutant 233/1 C5, isolate 233 WT, mutant 233/1 and the recipient strain 233/1 C5) on Fusarium wilt of carnation (cv. Indios)[z]

Strain	Disease index (0-100)		
	Experiment 1	Experiment 2	Experiment 3
none	83.7 d	65.0 e	65.0 c
233 WT	16.2 ab	2.5 ab	0 a
233/1	21.2 a-c	1.2 a	10.0 ab
233/1 C5	23.7 a-c	10.0 a-c	21.2 b
T26.1	10.0 a	26.2 cd	16.2 ab
T26.6	15.0 ab	22.5 b-d	15.0 ab
T26.19	43.7 c	26.2 cd	17.5 ab
T26.40	20.0 a-c	16.2 a-d	8.7 ab
T27.7	17.5 ab	15.0 a-d	28.7 b
T27.11	30.0 a-c	50.0 e	8.7 ab
T27.28	38.7 bc	21.2 a-d	27.5 b
T28.9	17.5 ab	23.7 b-d	16.2 ab
T28.26	35.0 a-c	31.2 d	23.7 b

[z] See Table 1.

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Simultaneous in situ detection of a *Pseudomonas fluorescens* strain and the total bacterial population on barley roots by dual staining with fluorescence-labeled antibody and rRNA-targeted oligonucleotide using confocal laser scanning microscopy

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Abstract

Pseudomonas fluorescens strain DF57 was introduced into natural soil as a barley seed coating. The establishment and proliferation of the strain and other bacteria on the roots was followed by a strain-specific fluorescence-labeled antibody and a fluorescence-labeled 16S rDNA probe, EUB338, which is specific for the domain Bacteria. Our preliminary study indicated that DF57 became well established on 1-day-old roots, whereas, only few other Eubacteria were established at this time. After seven days, strain DF57 was rare, occuring as single cells or very few together, while many other soil bacteria had colonized the rhizoplane. The results indicate that strain DF57 showed poor colonization of the rhizoplane, possibly due to competition from other soil bacteria.

Introduction

The rhizosphere is a habitat for a variety of soil microorganisms with many different types of metabolism and adaptative responses (Sørensen 1997). The roots are colonized by micro-organisms, notably bacteria, which may depend completely on simple organic molecules exuded from the plant cells. This group of early-colonizing bacteria primarily includes rhizobacteria such as *Pseudomonas* spp. The bacteria are anticipated to compete strongly with each other and with symbionts and pathogens during the early phase of root colonization. Colonization sometimes involves a chemotactic response by the bacteria to compounds exuded by the root, active movement towards and along the root surface, and specific molecular recognition during attachment. The bacteria are distributed in clumps or patches on the roots, interspersed by areas where they are sparse or absent.

With the introduction of effective biological control agents (BCAs) in agriculture there is a need to develop methods to obtain information about the establishment, proliferation and activity of the BCAs under natural condition before selecting those strains that are most suited for application (Assmus et al. 1997, Lübeck et al. 1998). In situ localization of bacteria on root surfaces using confocal laser scanning microscopy (CLSM) with fluorescence-labeled antibodies or rRNA-targeted oligonucleotides are methods that provide detailed information on root colonization (Assmus et al. 1995, 1997, Hansen et al. 1997, Macnaughton et al. 1996, Schloter et al. 1993). Assmus et al. (1997) described a technique using dual staining with fluorescence-labeled antibodies and rRNA-targeted oligonucleotides. This combined staining technique has the advantages of enabling the study of specific strains, species or populations of different species in their natural environment as visualized with CLSM. When used

simultaneously, antibodies may specifically detect the strain being monitored, and the oligonucleotide probes can localize other bacteria (Assmus et al. 1997).

Our general goal is to study bacterial population dynamics on barley roots after seed-inoculation with specific *Pseudomonas* strain, using rRNA-targeted oligonucleotide probes and fluorescence-labeled antibodies recorded by CLSM. The investigation focuses on the diversity, distribution, and activity of root-colonizing bacteria over time. For detection and quantification of the different bacteria, a combination of strain-specific fluorescence-labeled antibodies and species-/genus-specific rDNA probes labeled with different fluorescent compounds will be used. The *P. fluorescens* strain DF57 is used in the pilot studies where barley seeds inoculated with strain DF57 are placed in non-sterile soil. Root samples are collected after one and seven days to compare the establishment on the roots of strain DF57 and other bacteria over time.

Materials and Methods

Pseudomonas fluorescens DF57 was grown in Luria broth (LB) at 30 °C overnight on an orbital shaker at 225 rpm. The bacteria were centrifuged twice at 6,000 *g* for 7 min and resuspended in 0.9% NaCl before coating barley seeds. Prior to coating, non-sterilized barley seeds cv. Digger were pre-germinated between two sheets of moist filter paper overnight in darkness at room temperature. The pre-germinated seeds were then coated in bacterial suspension (0.9% NaCl) containing $5x10^9$ CFU/ml for 30 min at rpm. Bacterial seeds were placed on sterile 0.5% agar plates or placed in non-sterile soil (approx. 15% water). Roots from the agar plates were isolated after one day while roots from the soil were isolated after one day and seven days. The roots were washed in sterile phosphate-buffered saline (PBS, pH 7.2) followed by fixation in 4% paraformaldehyde/PBS for 4 h.

For detection, bacteria were first labeled with rhodamine-conjugated antibody according to Hansen et al. (1997), followed by labeling with the fluoresceine-marked Eubacteria probe EUB338 (Amann et al. 1990) as follows. The roots were washed in PBS and transferred to microtubes containing 0.5 ml pre-heated (50 °C) hybridization buffer (0.9 M NaCl, 0.01% SDS, 5 mM EDTA, 20 mM Tris-HCl, pH 7.0). A total of 180 ng EUB338 was added and the tubes were incubated at 50 °C at least 14 h in a hybridization oven. After hybridization, samples were washed for 1 min in the hybridization solution then washed twice in the same solution for 30 min at 50 °C. Finally, the roots were washed in PBS followed by sequential transfer through 25, 50, 75 and 90% glycerol of an 85% glycerol solution. Roots were mounted in antifading solution and observed with CLSM (TCS4d, Leica Laser Technik GmbH, Heidelberg, Germany) as described in Hansen et al. (1997).

Results and Discussion

In our initial studies, we inoculated pre-germinated barley seeds with *P. fluorescens* strain DF57, and placed the seeds on 0.5% water-agar plates at room temperature. After approximately 24 h, the roots were severed and prepared in different ways to achieve optimal detection. Fixation time, concentration of paraformaldehyde, hybridization time, and probe concentration were adjusted until the labeling procedure gave an acceptable signal (see Materials and Methods). Dual staining was introduced using the rRNA-targeted oligonucleotide EUB338 in combination with the fluorescence-labeled antibody against strain DF57.

In our subsequent work testing dual staining of bacteria on barley root surfaces recovered from natural non-sterilized soil, we studied the establishment and proliferation of strain DF57 on 1-day-old and 7-days-old roots, respectively. Simultaneously, we studied the distribution and quantity of other Eubacteria by the EUB338 probe. On 1-day-old roots, DF57 was well established as single cells or in small clumps containing a few cells along the entire root (Fig. 1). Few other bacteria of different types, as stained only by the EUB338 probe, were recognized dispersed along the roots. Morphologically distinct types of bacteria stained only by the EUB338 probe were observed. On 7-days-old roots we found only very few cells of strain DF57 on the rhizoplane but detected many other bacteria (Fig. 2).

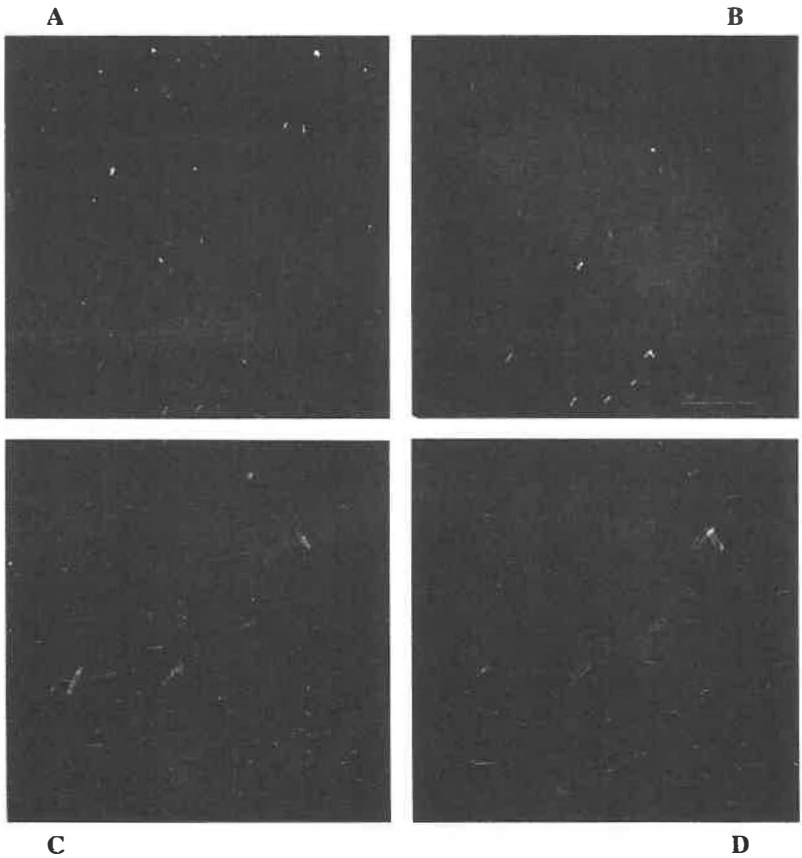

Fig. 1. Bacterial rhizoplane-colonization on one-day-old barley roots in non-sterile soil. (A) bacteria stained with fluorescein-labeled rDNA probe EUB338. (B) *P. fluorescens* strain DF57 stained with strain-specific rhodamine-labeled antibody. Comparison of A and B show that some bacteria are not stained with the rhodamine-conjugated antibody, whereas, all cells stained with the antibody are also labeled with EUB338. (C) Higher magnification of an area from A (zoom factor is 3) showing that EUB338 stains the interior of the cell, (D) while the antibody only stain the cell surface. Bar = 20 μm.

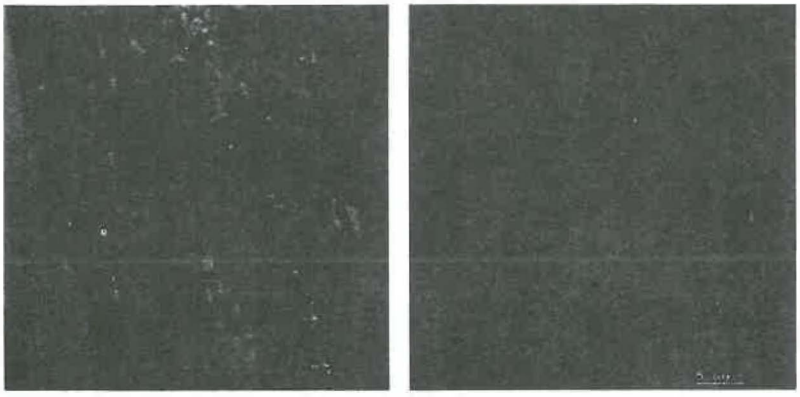

Fig. 2. Bacterial rhizoplane colonization on 7-days-old barley roots from non-sterile soil, (left) staining with EUB338. (Right) staining with antibody. Staining with EUB338 showed that the rhizoplane was well colonized with bacteria. None of the bacteria stained with antibody for *P. fluorescens* strain DF57. Bar = 5µm

The results show that we are able to study the establishment of bacteria at the rhizoplane, using in situ hybridization with rRNA-targeted oligonucleotide probes. The dual staining technique using a strain-specific antibody and an rRNA-targeted oligonucleotide probe, showed that *P. fluorescens* strain DF57 was unable to establish and proliferate on the rhizoplane, for a longer period of time. This could be due to strong competition from other soil bacteria, which were good root colonizers. Alternatively, strain DF57 may preferentially proliferate in the rhizosphere soil rather than the rhizoplane (Hansen et al. 1997).

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Development of specific PCR primers for monitoring a biocontrol strain of *Gliocladium roseum* in field soils

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Abstract

A strain of *Gliocladium roseum* strain IK726 has proven highly effective as a biocontrol agent in the field. Specific detection of this strain would facilitate evaluation of its establishment, proliferation and activity and is important for risk assessment. Using the PCR based fingerprinting method, UP-PCR, it was attempted to identify specific amplification products present in IK726, but not in other *G. roseum* strains. While this proved to be difficult using 21 primer combinations, another strategy was followed where similar amplification products present in IK726 and some of the other *G. roseum* strains were identified and isolated. Restriction fragment length polymorphism (RFLP) analysis of these products indicated sequence differences which may facilitate the development of specific primers. Sequencing of the products are being carried out.

Introduction

During the next decade biological control may become an important component of plant disease management. Seed treatment with a storable formulation of *G. roseum* strain IK726 has proven highly effective under field conditions for control of cereal diseases caused by seed-borne pathogen disease of cereals (Jensen et al. 1997, Knudsen et al. 1995). For development of effective and reliable biological control agents (BCAs) and in connection with risk assessment and product approval, there is a need for methods which can give information on establishment, proliferation and activity under natural conditions. In many instances deliberate release of genetically-modified organisms are not allowed. In such cases methods based on PCR might prove valuable for specific detection in seed, soil and root samples (Lübeck and Lübeck 1996).

PCR fingerprinting methods using primers without knowledge about the target sequences are unsuitable for direct detection of specific strains in complex environments such as soil. These methods require pure cultures because of the nature of the primers that allows amplification from almost any organisms. However, by identification of specific amplification products unique to the target organism, these methods can be used for the design of probes or specific PCR-primers (Lübeck and Lübeck 1996).

In an initial study, 'fingerprinting' with universally primed PCR (UP-PCR) (Bulat et al. 1990, Bulat and Mironenko 1996), was performed on IK726 and several other *G. roseum* strains from Danish soils. Results showed that almost all the *G. roseum* strains, including

IK726, were genetically very similar (Bulat et al. 1997, Lübeck et al. 1996).

The aims of this study were (i) to apply a wide range of specially designed UP primers in pairwise combinations to selected *G. roseum* strains, to identify specific amplification products, and (ii) use such products for constructing specific primers facilitating development of a PCR based diagnostic assay for detection of the IK726 strain.

Materials and Methods

Strains used in this study are listed in Table 1. DNA extraction was carried out as described in Lübeck et al. (1998) and Naumov et al. (1997). Elution of PCR products was carried out using the Quiax kit (Diagen GmbH, Düsseldorf, FRG) according to the manufacturer's instructions. Restriction digestions were carried out according to the manufacturer (GIBCO BRL, Life Technologies Inc., MD, USA).

Table 1. List of *Gliocladium* strains.

Strain no	Source and origin	References
GR1[Y]	Soil, Flakkebjerg, Denmark	1
GR2[Y]	Potato, Denmark	1
GR3[Y]	Soil, Annecy, France	1
GR4[Y]	Soil, Slagelse, Denmark	1
GR5[Y]	Soil, Taastrup, Denmark	1
GR6[Y]	Soil, Taastrup, Denmark	1
GR7[Y]	Soil, Taastrup, Denmark	1
GR8[Z]	Soil, Slagelse, Denmark	1
GR9[Z]	Soil, Slagelse, Denmark	1
GR10[Z]	Soil, Slagelse, Denmark	1
GR11[Z]	Soil, Slagelse, Denmark	1
GR12[Z]	Soil, Slagelse, Denmark	1
GR13[Z]	Soil, Slagelse, Denmark	1
IK726[Z]	Soil, Taastrup, Denmark	1,2

[Y] supplied by Ulf Thrane, [Z]supplied by Inge Knudsen. Strain IK726 is our selected antagonist.

UP-PCR was performed in 20 µl volume containing 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.4 mM dNTPs, 2.8 mM $MgCl_2$, 20 ng of each primer, between 20 and 200 ng of total DNA, and 2-3 units of *Tsp* (*Thermus* sp.) DNA polymerase (courtesy of Dr O.K. Kaboev, PNPI, Russia) and was carried out using the high ramping thermal cycler TC-1000M (PNPI, St. Petersburg, Russia) for 30 cycles using 0.5 ml narrow tubes (Lenmedpolimer, St. Petersburg, Russia). The cycling parameters were as follows (the time includes temperature changing periods): DNA denaturation at 92 °C for 50 s (first denaturation step at 94 °C for 2.5 min), primer annealing at 52-55 °C for 90 s and primer extension at 69 °C for 60 s. The rate of ramping between the different temperatures was about 4 °C/s. During the first 5 cycles, primer annealing time was extended to 120 s. The final extension step were performed at 69 °C for 3 min. Several DNA concentrations were tested in PCR in order to ensure the reliability of electrophoretic banding profiles.

Amplification products were initially tested on 1.7%, 7 cm long agarose gels at 150 V with cooled TBE buffer for 40 min, and then electrophoresed on 5.5%, 12 cm long polyacrylamide gel (PAAG) at 120 V for approximately 4 h or 5.5% 20 cm long PAAG at 170 V for

approximately 10 h with cold (about 12 °C) TBE buffer. The gels were stained with ethidium bromide and photographed in UV light.

The sequences of the UP primers used were:

3-2	(16 mer): 5'- TAAGGGCGGTGCCAGT-3' (Bulat et al., 1994)
AA2M2	(16 mer): 5'- CTGCGACCCAGAGCGG-3' (Lübeck et al., 1998)
AS4	(16 mer): 5'- TGTGGGCGCTCGACAC-3' (Lübeck et al., 1998)
AS15	(17 mer): 5'- GGCTAAGCGGTCGTTAC-3' (Bulat et al., 1994)
AS15inv	(17 mer): 5'- CATTGCTGGCGAATCGG-3' this study
L21	(21 mer): 5'- GGATCCGAGGGTGGCGGTTCT-3' (Naumov et al., 1997)
Fok1	(18 mer): 5'- GGATGACCCACCTCCTAC-3' (Lübeck et al., 1998)

Labeling of UP-PCR products was conducted as follows. A small aliquot (about 0.5 µl of 20 µl total volume) of total PCR products generated from one strain with one UP primer were re-amplified in the same conditions as above excepting the dCTP was replaced for (α-^{32}P) dCTP (40 µCi; 10 mCi/ml; 3000 Ci/mmol) (Izotop, St. Petersburg, Russia) and PCR was run for three cycles with the primer extension step for 10 min instead of 1 min. The reaction was terminated at 94 °C for 3 min and subsequently the labeled products were cooled on ice and further added to the hybridization solution.

Purified fragments and total UP-PCR products generated from strains with primer AS15inv were spotted onto nylon filters (Hybond N$^+$, Amersham, UK) according to the manufacturer's instructions. Hybridization of fixed DNA with the corresponding (α-^{32}P) dCTP labeled amplification products was conducted at 68 °C for 8 to 14 h as follows. First, the filter was prehybridized in 5 ml prehybridization solution (1% casein, 3% gelatine, 0.5 M NaCl, 0.1 M Tris HCl, pH 7.8 at 68 °C for 1 h, followed by hybridization using the same incubation temperature in 5 ml hybridization solution (composition of 20 ml: 1.8 ml of 5 M NaCl, 1.2 ml of 1 M Tris HCl (pH 7-8), 0.32 ml of 0.5 M EDTA, 0.55 ml of 3.6% solution of tetrasodium pyrophosphate $Na_4P_2O_7$, 1.5 ml of 10% SDS (Hybond N$^+$), 0.2 g heparin (ca. 25,000 units) with the labeled probe for minimum 8 h. Washing was performed at 68 °C for 20 min successively with 2X SSC (containing 0.1% SDS), 1.3X SSC (0.1% SDS), 0.8X SSC (0.1% SDS), and 0.1X SSC (0.1% SDS).

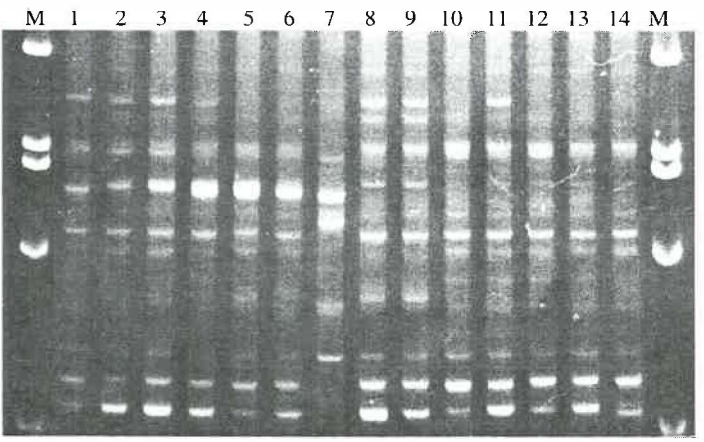
Results and Discussion

Seven UP primers in pair-wise combinations (total 21 primer combinations) were tested for the ability to distinguish the *G. roseum* strains with focus on the possible finding of markers for the antagonistic IK726 strain. After the first primer combination was applied to all strains, the strategy was to delimit the sample of strains by choosing five strains which had the most similar banding profile as IK726. These five strains and IK726 were tested by the remaining 20 primer combinations with focus on differentiating IK726 from the others. The screening of primer combinations resulted in the choice of the combinations which seemed most promising (Bulat et al., *unpublished data*). Subsequently these combinations were applied to all strains.

Although it was possible to distinguish all strains and to obtain a specific banding profile (fingerprint) for IK726 for some of the primer combinations (data not shown), it proved to be difficult to find specific amplification products (= markers) only present in IK726. Instead of using more primers and primer combinations, we decided to use another strategy. By selection

of a fragment in IK726 and corresponding fragments present in banding profiles of some but not all of the strains, sequence differences in these, if present, might then be used to design specific primers. This strategy is quite similar to the use of the ITS region of nuclear ribosomal DNA for development of specific primers; a strategy which successfully have proved valuable for species-specific detection (Lübeck and Lübeck 1996). The ITS approach may not be suitable for primer development for single strains since the spacer sequences likely possess no or inadequate differences among different strains from homogeneous species like *G. roseum*. We found earlier that all the fourteen strains included in this study possessed ITS1 with identical size as well as an identical restriction profile (Bulat et al. 1998). The feature of the UP-PCR technique is that the unique primer design attempt to target the priming to more variable areas (e.g., intergenic intervals) of the genome (Bulat and Mironenko 1996, Bulat et al. 1998) and therefore it may be a more convenient approach for identification of sequence variability to develop specific PCR primers for detection of individual strains.

A

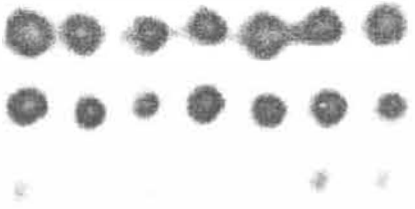


B

	IK726	GR12	GR10	GR13	GR5	GR3	GR2
0.9 kb	+	+	+	+	+	+	+
"large"		+	+	+	+		
"small"	+			+	+	+	
"Smallest"							+

Fig. 1. UP-PCR banding profiles for *Gliocladium roseum* strains generated with the UP primer combination Fok1/AS15inv. (A) Lane 1-14 *G. roseum* strains no. IK726, GR12, GR10, GR13, GR5, GR3, GR2, GR4, GR7, GR6, GR11, GR9, GR8, and GR1 respectively. (Borderlines) molecular weight marker (lambda phage DNA digested with *Pst*1). 5.5% acrylamide gel was used. (B) Scheme showing which strains contain the 0.9 kb fragments. "large" indicate that the 0.9 kb fragments for these strains are slightly larger than the 0.9 kb fragments from other strains, which in contrast are indicated as "small".

By evaluating the digestion profiles, the primer combinations AS15/AS15inv and FokI/AS15inv both were found to give one sharp band at about 0.9 kb in seven of the strains (Bulat et al. unpublished data). It was later found that these fragments were amplified with primer AS15inv alone. Running the profiles on agarose gels, the 0.9 kb fragments seemed to be of similar size, while on PAAG it was possible to recognize slight differences in size (Fig. 1). The 0.9 kb fragments from the seven strains were eluted and subsequently tested in a dot blot hybridization experiment to study the sequence similarity of the fragments. All proved to be cross-homologous. In contrast, the whole UP-PCR profiles for the remaining seven strains did not contain homologous DNA and gave very weak signals (Fig. 2).



IK726 GR13 GR5 GR3 GR12 GR10 GR2 - 0.9 kb fragment (AS15inv)
IK726 GR13 GR5 GR3 GR12 GR10 GR - UP-PCR products (FokI/AS15inv)
GR4 GR7 GR6 GR11 GR9 GR8 GR1 - UP-PCR products (FokI/AS15inv)

Fig. 2. Dot blot hybridization of the 0.9 kb isolated fragments. Autoradiography showing the dot blot from the cross-hybridization experiment using the isolated 0.9 kb fragment from IK726 as labeled probe. As indicated below the picture, the first row of dots are the 0.9 kb fragments from the respective strains, the second row of dots are the total UP-PCR profile generated with the primer combination FokI/AS15inv from the same strains as the first row, while the third row are the total UP-PCR profile from the remaining strains.

To analyze the fragments further, restriction fragment length polymorphism (RFLP) analysis were carried out. Thirty-one restriction enzymes was applied to one of the fragments (from strain GR12), and from this four enzymes, *Bgl*II, *Dra*I, *Hinc*II, and *Nde*I, digesting the fragment once were selected. These four restriction enzymes were thus applied to all seven fragments, and it was found that only *Hinc*II cut all seven fragments resulting in two bands with similar size, while *Bgl*II, *Dra*I and *Nde*I digestions showed that some of the fragments did not contain the recognition sequence for these enzymes (Table 2).

We are at present attempting to sequence the seven fragments in order to find sequence variability facilitating the development of specific primers. Direct sequencing of the fragments were not possible using the primer AS15inv alone because it anneals to both ends of the fragments, and were also found to anneal weakly internal in the fragment. Therefore to perform direct sequencing it was necessary to use other strategies. By finding a restriction enzyme that cut the fragments into two pieces, one strategy might then be to use the AS15inv primer as sequencing primer for the part of fragment which do not have the internal AS15inv

site. It might not be necessary to sequence the 0.9 kb fragment in its entirety in order to reveal sequence differences. Another strategy for sequencing would be to attach linkers to the fragments, but unfortunately the enzyme *Hinc*II which is able to cut all seven fragments creates blunt ends which make ligation of linkers more difficult. Instead *Bgl*II or *Nde*I, being able to cut six of the seven fragments (Table 2), can be used.

Table 2. Restriction digestions of the isolated 0.9 kb fragments.

Strain	*Hinc*II	*Bgl*II	*Nde*I	*Dra*II
IK726	+	+	+	-
GR2	+	-	-	-
GR3	+	+	+	-
GR5	+	+	+	+
GR10	+	+	+	+
GR12	+	+	+	+
GR13	+	+	+	+

+ = restriction enzyme digested the fragment from the respective strains once.
- = restriction enzyme did not digest the fragment from the respective strains.

Although we did not finish the development of specific primers yet, the preliminary results from this study seem promising. The fragments obtained from seven strains are cross-homologous. All can be digested with *Hinc*II creating bands of similar sizes, thus allowing alignment of the sequences. Most interestingly we observed sequence polymorphisms by the RFLP analysis using only four restriction enzymes (Table 2). Therefore, it seems likely that sequencing and subsequent alignment of the fragments may reveal some sequence differences facilitating the design of specific primers for IK726, that are directed to the area(s) with "unique" sequences.

If so, the primers are going to be tested for their specificity using a variety of *G. roseum* strains as well as a representative amount of other fungi isolated from soil. If the specificity using pure cultures prove to be adequate, different assays for monitoring the biocontrol agent IK726 in field experiments will be developed. One approach might be detection of IK726 from soil, rhizosphere, roots and seeds based on extraction of the total fraction of DNA. Other approaches might be detection of IK726 directly on seeds and roots by in situ PCR or by in situ hybridization using one of the specific primers as probe.

This study may illustrate a convenient approach to developing molecular tools for detection of individual fungal strains without previously having information of sequences from any genes in the target organisms.

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Benomyl resistance as a marker in ecological studies of fungi introduced into the environment

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Abstract

The use of UV-c induced benomyl resistance was studied for four different fungal species. An extensive screening procedure was necessary to select suitable strains. In general, resistance-labeled strains were used successfully in ecological experiments. One strain that passed the in vitro selection procedure, showed a loss of resistance in plants. Pitfalls in using benomyl resistance markers can be a complex inheritance of the characteristic and changes in ecological properties that can not be detected with in vitro screening of mutants.

Introduction

To study the ecology of fungi introduced in non-sterile habitats, it is necessary to distinguish the strain applied from the resident microbial population. Analysis of factors that influence the establishment and population dynamics of introduced fungi, including soil type, temperature, moisture, propagule type, and formulation, is only possible if a suitable detection technique is available. Furthermore, a detection system will facilitate the investigations on the mechanisms of the action of antagonists against plant pathogens, thus improving the reliability of biocontrol with microbial antagonists. Certainly in case of failure of a biocontrol experiment, data on the population dynamics of the antagonist are indispensable for a correct understanding of the situation. A way to overcome the problem of detection and enumeration of introduced fungi is the use of genetic markers, such as resistance to the fungicide benomyl.

Resistance to benomyl has been proven to be a useful selective marker for several fungal species (Couteaudier and Alabouvette 1990, Papavizas and Lewis 1983, Sivan and Chet 1989). However, benomyl-resistant mutants have been found with changed antagonistic activities (Sivan and Chet 1989), or an altered virulence pattern (Kroon and Elgersma 1991). Therefore, we adapted a strict selection and testing procedure for benomyl mutants.

Materials and Methods

Strains of *Trichoderma harzianum*, pathogenic and non-pathogenic *Fusarium oxysporum*, *Verticillium biguttatum,* and *Arthrobotrys oligospora* were used in this study. Benomyl resistance was induced by UV-c irradiation of conidia plated on potato dextrose agar (PDA) containing 10 mg benomyl (Benlate, 50% a.i.). Onto each agar plate (9 cm diameter), 10^6 to 10^7 conidia were spread and irradiated with an UV source (Philips, Eindhoven, The Netherlands, 15 W 253.7 nm) at 70 cm, for 1 to 90 min (Postma and Luttikholt, 1993). Colonies growing on the benomyl amended PDA were transferred for further testing.

Mutant strains were subjected to a screening procedure that considered colony morphology, growth rate, stability of the mutation, percentage benomyl resistance of spores, and in vitro antagonistic activity when applicable. Genetic stability of benomyl resistance was checked by plating conidia on medium with and without benomyl. The percentage benomyl resistance was expressed as the ratio of colony numbers on benomyl amended medium and benomyl-free medium, multiplied by 100%. A further check on the stability of the selected mutant strain of *F. oxysporum* f.sp. *dianthi*, a pathogen of carnation, was performed after the fungus had infected plants. The fungus was applied to soil in which carnation cuttings were planted (Postma and Rattink 1992). After 140 days, it was re-isolated from the stems of carnations, showing various levels of disease symptoms. The level of benomyl resistance of the reisolated pathogen was determined by comparing number of colony-forming units (CFU) on semi-selective medium with and without benomyl.

Enumeration of CFU was carried out by dilution plating on semi-selective media (Postma and Rattink 1992). For *F. oxysporum*, the fungicides dichloran and fenaminosulf were used to obtain a suitable selective medium (Postma and Rattink 1992). In the semi-selective medium for *T. harzianum*, the combination of the fungicide furulaxyl and the microbial growth inhibitors propionic acid and Triton X-100 resulted in a strong growth reduction of rapidly spreading fungi. Colonies of *T. harzianum* could be easily distinguished and counted on this medium (Kok et al. 1996).

The semi-selective media developed were tested for suitability for enumeration of benomyl-resistant mutants of *A. oligospora*. Outgrowth of *A. oligospora* into non-sterile soil was studied using the method of Stirling and Mani (1995) as alternative to soil dilution plating. Alginate prills containing spores and several amendments were placed on glass slides and covered with nylon bolting cloth (60 µm mesh). The slides were placed in a Petri dish and covered with non-sterile soil. After two weeks, the slides were recovered and the outgrowth of the fungus was determined under the microscope, using a rectangle ocular grid. The amendments tested were benomyl (50 mg a.i. per liter formulation) and wheat bran as a nutrient source (10 g bran per liter formulation).

Results

In the in vitro selection procedure the major part of the benomyl-resistant mutants were rejected. Table 1 shows the results of the selection procedure of *F. oxysporum* f.sp. *dianthi*. Similar results were obtained with *T. harzianum* and *A. oligospora*. No benomyl-resistant mutants were observed for *V. biguttatum*.

Table 1. Selection of benomyl-resistant mutants of pathogenic *F. oxysporum* f.sp. *dianthi*

Step of the selection procedure	Number of isolates recovered
After UV-irradiation on benomyl medium	102
Sufficient growth on benomyl medium	95
Same morphology as wild type	81
20% of conidia benomyl resistant	10
100% of conidia benomyl resistant	4
Stable marker on agar	2
Pathogenic after stem inoculation	1

Benomyl resistance was not always inherited in a genetically stable way. For example, in *T. harzianum,* monospore isolates were produced, that yielded 100% benomyl resistant spores. Monospore isolates obtained from these strains, however, yielded spores with different levels of benomyl resistance (Table 2). The strains of the second generation of monospore isolates that yielded 100% benomyl resistant spores, were found to be genetically stable. In pathogenic *F. oxysporum* f.sp. *dianthi,* benomyl resistance in a mutant strain was reduced after reisolation of the fungus from plants, and the percentage of benomyl-resistant strains recovered was lower in plants with a high disease severity index (Table 3).

Table 2. Benomyl resistance of spores from monospore isolates from 100% benomyl resistant parent strains of *T. harzianum*

Benomyl resistant spores (%)	Number of isolates
0-20	5
20-60	18
60-90	3
100	2

Table 3. Benomyl resistance of pathogenic *F. oxysporum* f.sp. *dianthi* colonies, after reisolation from plants with different level of disease

Disease severity index	Benomyl resistance (%)
1	86.0
4	2.0
5	0.7

Growth of *A. oligospora* was greatly reduced on two media amended with a combination of inhibiting compounds. No medium was found on which enumeration of this fungus was possible, due to the slow growth rate of the fungus and its inconspicuous colony morphology. Fast spreading, benomyl-tolerant fungi like *Mucor* tended to overgrow the soil dilution plates before reliable counts of *A. oligospora* could be made. Direct observation of outgrowth into soil however was found to be a suitable alternative for experiments on the effects of formulation on fungal establishment in soil. Alginate formulations with amendments of benomyl and wheat bran enabled *A. oligospora* to grow into non-sterile soil (Table 4).

Table 4. Effect of additives on the outgrowth (length of hyphae growing from alginate prills) of a benomyl-resistant mutant of *A. oligospora* from alginate pellets in non-sterile soil after two weeks incubation

	Outgrowth (mm)	
Treatment	Median	Maximum
Control	< 0.1	0.5
Bran	3.6	9.9
Benomyl	3.3	8.0
Benomyl plus bran	2.0	9.3

Discussion

The usefulness of benomyl-resistant mutants in ecological research on fungi introduced into soil has been demonstrated in many systems. In our laboratory, benomyl markers were applied for the assessment of formulation effects on the population dynamics and biocontrol efficacy of *T. harzianum* (Kok et al. 1996), and for studying the population dynamics and interaction of pathogenic and non-pathogenic *F. oxysporum* f. sp. *dianthi* in carnation (Postma and Luttikholt 1993, 1996, Postma and Rattink 1992). The strategy of inducing benomyl resistant-mutants by UV-c radiation is fast, uncomplicated, and inexpensive. However, for reasons unknown to us, no benomyl-resistant mutants of *V. biguttatum* were formed so this technique may be limited to certain fungi.

A strict selection procedure for mutants was applied, since random mutagenesis with UV-c can lead to unwanted loss of fitness and other strain characteristics. Indeed, most mutants were rejected in the in vitro selection procedure. Special attention is needed to the genetical stability of the mutants, because it was found that the percentage benomyl-resistant spores could decrease, even in strains derived from monospore isolates that yield 100% benomyl resistant spores. Even after the extensive in vitro screening, one of our mutant strains showed loss of benomyl resistance after reisolation from plants. Furthermore, loss of resistance was correlated with high disease index, suggesting some link between benomyl-resistance and virulence of this pathogenic isolate. In another mutant strain of pathogenic *F. oxysporum* no such link was found (Couteaudier and Alabouvette 1990).

Benomyl-resistance itself is not selective enough for dilution plating. Extra inhibitors are necessary to suppress growth of benomyl-tolerant fungi. *Arthrobotrys oligospora* showed a high sensitivity towards the additional inhibitors in the semi-selective media, and we were not able to use soil dilution plating for this fungus. Direct observation of growth in soil of this fungus gave information on the effect of formulation on the establishment in soil. An inhibition of the benomyl-sensitive part of the resident fungal community or addition of extra nutrients in the formulation enabled the spores to overcome the soil fungistasis and to grow from the alginate prills. However, the direct observation technique can not be used in experiments in which population dynamics of the antagonist and the resulting control effects are studied simultaneously.

Our conclusion is that UV-c induced benomyl resistance is a suitable marker in ecological studies on introduced fungi. However, this strategy does not work for all fungi. Pitfalls in using benomyl resistance markers can be a complex inheritance of the characteristic and changes in ecological properties that can not be detected with in vitro screening of mutants.

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Development of specific primers for monitoring *Gliocladium roseum* in field soils

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Abstract

During the next decade biological control may become an important component of plant disease management. A storable formulation of *Gliocladium roseum* (IK726) has proven highly efficient against seed-borne disease of cereals under field conditions when applied as a seed treatment. For development of efficient and reliable biological control agents (BCAs) and in connection with risk assessment and product approval, there is a need for methds which can give information on establishment, proliferation and activity of BCAs under natural conditions. In many instances deliberate release of genetic modified organisms are not allowed and in such cases methods based on PCR can be used for specific detection in seed, soil and root samples. The objective of the project is to develop a PCR based diagnostic assay for the detection of isolate IK726 by constructing specific primers.

PCR fingerprinting methods using primers without knowledge of the target sequences are unsuitable for direct detection of specific isolates in complex environments such as soil. These methods require pure cultures due to the nature of the primers that allow amplification products unique to the target organism, these methods can be used for the design of probes or specific PCR-primers. The fingerprinting method, universally primed PCR (UP-PCR), was performed on IK726 and several other *G. roseum* isolates from Danish soils using a wide range of specially designed UP primers, singly and in pairwise combinations. Although it was possible to distinguish the isolates and obtain a specific banding profile (fingerprint) for IK726, it proved to be difficult to find specific amplification products present only in IK726. However, one PCR product specific to IK726 and two other isolates out of fourteen *G. roseum* isolates tested was isolated. In order to differentiate IK726 from the two other isolates, specific PCR fragments only present in the latter isolates were also identified and isolated. Sequencing of these fragments are at present carried out in order to design specific primers that selectively amplify the fragments. These primers will be tested for their specificity using a variety of *G. roseum* isolates and a representative amount of other fungi isolated from soil. If the specificity using pure cultures prove to be sensitive enough different assays for monitoring the biocontrol agent IK726 in field experiments will be developed. One approach will be detection of IK726 from soil, rhizosphere, roots and seeds based on extraction of the total fraction of DNA. Another approach will be *in situ* detection of IK726 directly on seeds and roots.

Approaches to improve biocontrol

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Improving biocontrol activity of *Trichoderma harzianum* by molecular biology techniques

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Abstract

Biological control of plant pathogens is considered the most cost-effective and environmentally safe alternative for the acute problem of damage caused to crops by plant pathogens. Potential agents for biocontrol activity are rhizosphere-competent fungi and bacteria that exhibit antagonistic activity towards soil-borne plant pathogens. *Trichoderma harzianum* is a species comprised of few strains, each exhibiting effective antagonism of different degree on a variety of fungal plant pathogens. *Rhizoctonia solani*, *Sclerotium rolfsii* and species of *Pythium* are among the many pathogens that *T. harzianum* parasitizes. These pathogenic fungi cause diseases, seed rot and seedling damping-off of a variety of crops (Chet 1987, 1990).

Introduction

Trichoderma harzianum preparations are already used as biocontrol agents in soils and phyllosphere, but we still face the challenge of improving the existing biocontrol strains of *Trichoderma* in order to make plant-disease biocontrol a more effective and economical process. This goal can be achieved only if we acquire detailed knowledge of the mycoparasitic interaction and at the same time develop better molecular tools for genetic manipulations of *Trichoderma*.

In recent years progress has been made in elucidating the molecular basis of the mycoparasitic process. Several genes encoding lytic enzymes of *T. harzianum* have been cloned and characterized (Carsolio et al. 1994, De La Cruz et al. 1995, Garcia et al. 1994, Hayes et al. 1994, Limon et al. 1995, Lora et al. 1995, Peterbauer et al. 1996). This progress will facilitate the search for ways to improve the biocontrol capacity of *T. harzianum*.

We, among other labs, are investigating the different steps of mycoparasitism. Our efforts have been focused on two major steps of the process: The recognition and contact events, and characterization of the lytic enzymes secreted by *Trichoderma* in response to fungal hosts.

Recognition and contact of the host fungi by *T. harzianum*

Mycoparasitism is a complex process that involves "recognition" of the host, attachment and de-novo synthesis of enzymes that aid the parasite in penetrating the host and completing its destruction. The destructive parasitic process in *Trichoderma* is initiated by the attraction and direct growth of *Trichoderma* towards its host. Positive chemotropism was observed in *Trichoderma* (Chet et al. 1981), whereby it detected its fungal host from a distance and began to branch in an atypical manner towards it. The nature of the attractant or attractants has not been revealed so far. Subsequently, contact is achieved between the two fungi and as a result, *Trichoderma* coils around or grows along the host hyphae (Benhamou and Chet 1993, 1996,

1997, Elad et al. 1983). To aid its penetration of the host, *T. harzianum* forms hook-like structures that are characteristic to mycoparasitism (Elad et al. 1983).

The ability of different isolates of *Trichoderma* to attack *S. rolfsii* was correlated with the agglutination of *Trichoderma* conidia by *S. rolfsii* agglutinin (Barak et al. 1985). This suggested that lectins play a role in the interaction and recognition between *Trichoderma* and soilborne pathogenic fungi. To further test this possibility, Inbar and Chet (1992) designed a system based on the covalent binding of lectins to the surface of nylon fibers that mimic (in diameter and shape) the host fungus hyphae. They demonstrated the attachment and coiling of *T. harzianum* to nylon fibers that were coated with ConA or with LSR, that are lectins extracted from plants and *S. rolfsii*, respectively. *Trichoderma harzianum* did not make close contact nor coil around the untreated nylon fibers (Inbar and Chet 1992).

Later, Inbar and Chet (1994) isolated and purified a novel, 45 kDa lectin from *S. rolfsii* and used it to coat nylon fibers in the biomimetic system described before. They demonstrated that *T. harzianum* behaved towards the coated fibers exactly in the way it did towards a pathogenic host: it branched towards the fibers, it grew along and eventually coiled around them (Fig. 1). The coiling around the coated fibers was significantly more frequent than around uncoated fibers or fibers that were treated with non-agglutinating extracellular proteins from *S. rolfsii*. The lectin-mediated contact also elicited the higher expression of CHIT102, as discussed below. Although it is so far unclear whether other factors produced by either *T. harzianum* or *S. rolfsii* play a role in this interaction, the described experiments prove the role of lectins in mediating the contact between *Trichoderma* and *S. rolfsii*. Moreover, this contact initiated a signal transduction cascade towards the second most important step of mycoparasitism, the induction of lytic enzymes.

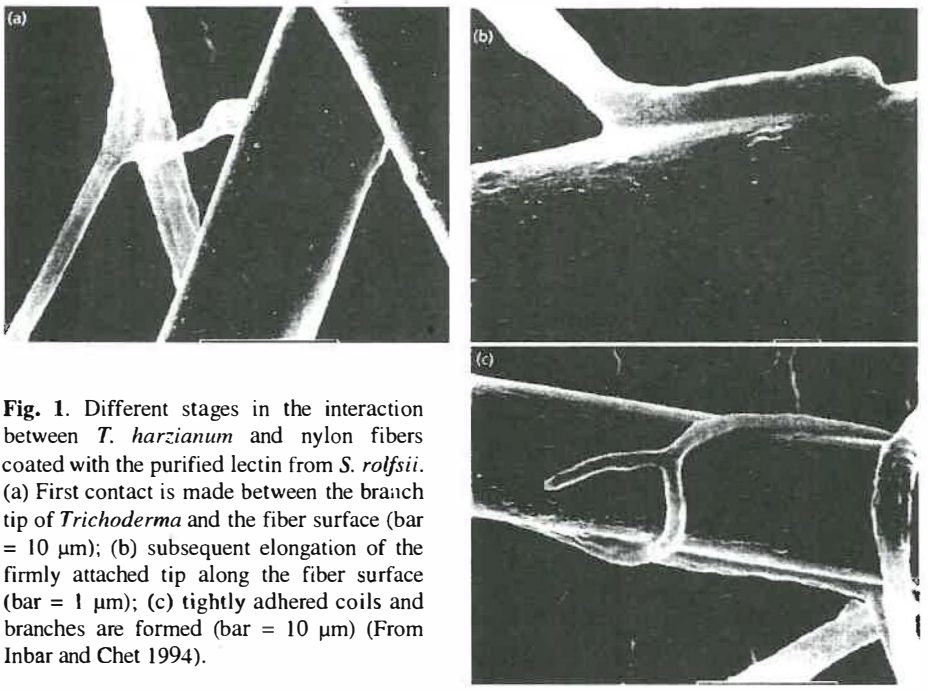

Fig. 1. Different stages in the interaction between *T. harzianum* and nylon fibers coated with the purified lectin from *S. rolfsii*. (a) First contact is made between the branch tip of *Trichoderma* and the fiber surface (bar = 10 μm); (b) subsequent elongation of the firmly attached tip along the fiber surface (bar = 1 μm); (c) tightly adhered coils and branches are formed (bar = 10 μm) (From Inbar and Chet 1994).

Lytic enzymes produced by *T. harzianum* in the mycoparasitic process

The main mechanism involved in the antagonism of pathogenic fungi by *T. harzianum* is the high production and secretion of lytic enzymes. Upon growth in induced conditions (i.e., in interaction with fungal hosts or in medium with chitin as a sole carbon source), *Trichoderma* secretes lytic enzymes, mainly chitinases and glucanases. These enzymes are active in penetrating the host cell walls and degrading them. Inbar and Chet (1995) investigated the chitinases secreted by *T. harzianum* that was grown in dual-culture with *S. rolfsii*. They demonstrated that the glucosaminidase, CHIT 102, was expressed at a low basal level before contact was made between the two fungi. The activity of CHIT 102 was strongly induced at the earlier stages of contact, and totally vanished at latter stages (Fig. 2). Strong activity of CHIT 73 could be detected at that time. An induction of CHIT 102 was also detected by the authors in the biomimetic system, where they demonstrated the attachment and coiling of *T. harzianum* around nylon fibers that were coated with a purified lectin from *S. rolfsii* (Fig. 3). These results demonstrate that CHIT 102 is apparently the first active chitinase in the interaction of *T. harzianum* with *S. rolfsii*.

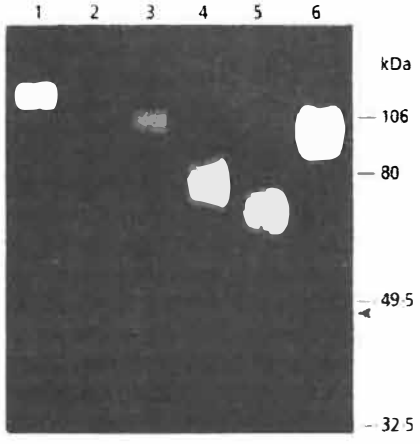

Fig. 2. Profile of chitinolytic enzymes (50 µg per lane) obtained from a dual culture of *T. harzianum* and *S. rolfsii* grown on synthetic medium (SM) supplemented with glucose (0.2%, w/v). Chitinolytic activity was detected following SDS-PAGE (10% acrylamide) using 4-MU-(GlcNAc)2 as the substrate. Lanes: 1, proteins produced by *S. rolfsii* before coming into contact with *T. harzianum*; 2, proteins produced by *T. harzianum* before contacting *S. rolfsii*; 3, 4 and 5, proteins obtained from the interaction zone at 12 h, 24 h and 48 h post-contact, respectively; 6, proteins produced by *T. harzianum* after 48 h incubation on solid SM with chitin as sole carbon source. The arrow indicates the position of CHIT 42 which is hardly visible in this photograph (From Inbar and Chet 1995).

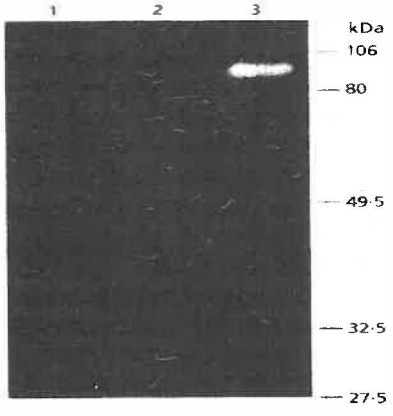

Fig. 3. Detection of chitinolytic activity in proteins (25 µg per lane) produced by *T. harzianum* grown in the biomimetic system. Lanes: 1, proteins obtained from *T. harzianum* grown on SM without nylon fibers; 2, proteins obtained from *T. harzianum* grown on SM in the presence of untreated nylon fibers; 3, proteins obtained from *T. harzianum* grown in the presence of nylon fibers coated with a purified lectin from *S. rolfsii*. Detection was carried out with 4-MU-(GlcNAc)2 as the substrate (from Inbar and Chet 1995).

Trichoderma harzianum secretes six chitinases, two N-acetylglucosaminidases, (CHIT 102 and CHIT 73), and four endochitinases (CHIT 52, CHIT 42, CHIT 33 and CHIT 31) (reviewed by Haran et al. 1996). CHIT 52 and CHIT 73 were first described by Haran et al. (1995). Using SDS-PAGE and a set of three fluorescent oligo-substrates that could distinguish between endochitinases and 1,4-β-N-acetylglucosaminidases, the authors demonstrated the induced activity of the different chitinases secreted by *T. harzianum* (Fig 4). In these experiments it was verified that CHIT 102 was the only chitinase of *T. harzianum* to be expressed constitutively (manifested by very low activity in growth with glucose; *data not presented here*). The activity of all the other chitinases was detected only upon induction (in growth with chitin as the sole carbon source), which indicated that they are expressed only in induced conditions. This fact led to the belief that if chitinases will be engineered in *T. harzianum* to be expressed constitutively, it will improve its mycoparasitic performance. To test this hypothesis, Haran et al. (1993) cloned the chitinase gene of *Serratia marcescens* (*ChiA*) under the constitutive 35S promoter of CaMV and introduced it into the *Trichoderma* genome. Two transformants were checked for chitinase activity. Both exhibited an average of 10 fold increase, compared to the wild type, in growth with glucose (non inductive conditions). Under growth with chitin as the sole carbon source, however, the transformants did not exhibit higher chitinase activity compared with the wild-type strain. When the extracellular chitinases of the transformants were analyzed, it appeared that for an unknown reason, the 58 kDa *Serratia* chitinases that were produced by both transformants in inductive growth conditions, were cleaved into two polypeptides, 40 and 18 kDa in size. Both polypeptides reacted with polyclonal antibodies raised against ChiA. The authors suggested that the high production of the *Serratia* chitinase, and its subsequent cleavage and secretion, probably interfered with the efficient secretion of the native chitinases, leading to their lower performance in culture. The parasitic performance of the *Trichoderma* transformants, as checked in dual cultures with *S. rolfsii*, was not affected by the cleavage of the *Serratia* chitinase. On the contrary, the transformants showed some advantage in their reaction with *S. rolfsii*. This was clear by the wider lytic zones that the transformants caused, compared to those the wild type did (Haran et al. 1993). A future strategy will be to clone a *Trichoderma* chitinase under a constitutive promoter and use it to replace the respective gene in the Trichoderma genome. This might prevent problems of incompatibility and still improve the performance of *Trichoderma* in bioassays.

A different approach to improve the parasitic performance of *T. harzianum* was used by Geremia et al. (1993) and Flores et al. (1997). They isolated the gene *prb1* that encodes a basic proteinase. The *prb1* expression was induced when *T. harzianum* was grown with the pathogen *R. solani*. This induction indicated that PRB1 is active in mycoparasitism. The authors transformed *T. harzianum* with high copy-number of *prb1* that contained its native promoter. In greenhouse experiments carried with cotton seedlings, they demonstrated that transformants that carried from one to five extra-copies of the gene were more efficient than the wild-type in preventing disease by *R. solani*. This *Trichoderma* strain was also very active against plant-pathogenic nematodes (I. Chet *personal communication*).

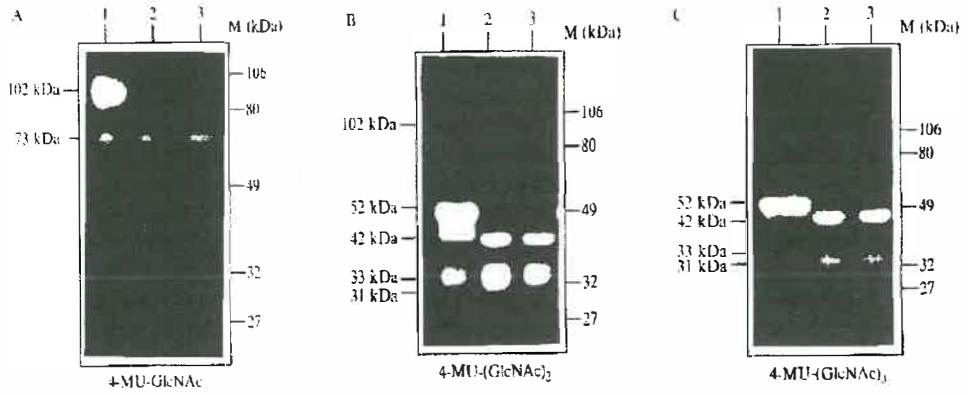

Fig. 4. Detection of extracellular chitinolytic activity of proteins produced by *T. harzianum* strain TM when grown on chitin as the sole carbon source. All lanes contain 20 µg extracellular protein, renatured following their separation by SDS-PAGE. Temperature treatments prior to loading on the gels were: lanes 1 in all panels = room temperature; lanes 2 = 3 min at 55 °C; lanes 3 = 3 min. at 100 °C. Chitinolytic activity was detected using in (A) 4-MU-(GlcNAc); (B) 4-MU-(GlcNAc)2 and (C) 4-MU-(GlcNac)3 as substrates (From Haran et al. 1995).

Glucanases secreted by *Trichoderma* were also found to be active in the lysis of several phytopathogenic fungi (Elad et al. 1982). *Pythium* spp. belong to the oomycetes, which are exceptional in that their cell walls contain cellulose instead of chitin as major structural component, and β-(1,3)-(1,6)-glucans (Blascheck et al. 1992). It is thus a preferred system to study the role of glucanases in mycoparasitism. Benhamou and Chet (1997) studied the parasitic interaction of *T. harzianum* with *Pythium ultimum* using electron microscopy and localization of glucanases produced by *Trichoderma*. They demonstrated the massive attack of β-1,3-glucanases on the cell walls of *Pythium*. By the sixth day after inoculation of the two fungi, the *Pythium* cells appeared completely degraded, while *Trichoderma* colonized heavily the host hyphae, occupying spaces where originally the host cytoplasm resided (Benhamou and Chet, 1997).

In attempt to further study the importance of glucanases in the mycoparasitic interaction of *T. harzianum* with *Pythium* and other phytopathogenic fungi, we have cloned and characterized an exoglucanase of *T. harzianum* strain T-Y. The enzyme was detected as a 110 kDa active protein in SDS-PAGE gels. The expression of the gene, encoding this glucanase, was low under non-inductive growth conditions. Its expression (detected by mRNA levels) in growth with laminarin as a sole carbon source was strongly induced, suggesting a possible role in mycoparasitism (R. Cohen-Kupiec, D. Friesem, K. Broglie, R. Broglie, and I. Chet *personal communication*).

In summary, although trials to manipulate the expression of lytic enzymes are preliminary, the positive results that have been obtained are promising. A joint effort should be made to develop strains of *T. harzianum* in which few genes that encode different lytic enzymes have been manipulated to be over expressed or constitutively expressed. The co-expression of such genes can lead to synergistic effects that will result in better *Trichoderma* strains.

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Potential for exploitation of ATP-binding cassette (ABC) transporters in biological control

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Abstract

ATP-binding cassette (ABC) transporters are membrane glycoproteins which utilize the energy derived from hydrolysis of ATP to drive the transport of endogenous metabolites and toxic xenobiotics through biological membranes. Their basic structure includes an hydrophilic moiety with a conserved domain and a moiety with several stretches of hydrophobic amino acids which are supposed to constitute transmembrane domains. Overexpression of some ABC transporters determines simultaneous resistance to many chemically unrelated compounds whereas disruption of genes encoding ABC transporters is associated with increased sensitivity to the same compounds. The mechanism of resistance conferred by overexpression of ABC transporters relies on increased energy-dependent efflux which, in turn, causes decreased intracellular accumulation of toxicants. Recently, we isolated the ABC transporter-encoding genes *atrA* and *atrB* from the filamentous fungus *Aspergillus nidulans* and *bc-atrA* from the plant pathogen *Botrytis cinerea*. We found that transcription of these genes is strongly enhanced within minutes following treatment with several antibiotics, plant defense compounds and fungicides. We hypothesize that some ABC transporters may have a role in secretion of compounds with antibiotic activity in soil. To test this hypothesis we put the cDNA of *atrB* in a yeast mutant disrupted in *PDR5*, a gene that encodes a well characterized ABC transporter conferring drug hypersensitivity upon disruption, under the control of the galactose-inducible *GAL10* promoter. We demonstrated that in permissive conditions transcription of *atrB* is induced and drug hypersensitivity partially restored. Currently, we are testing the possibility that the transporter encoded by *atrB* may transport compounds involved in antagonistic interactions of soil micro-organisms. Based on these findings we hypothesize that ABC transporters may find applications in plant disease control. One possibility is the use of selective inhibitors of ABC transporters to enhance activity of natural or synthetic fungitoxic compounds. We found that the inhibitory effect of some fungicides (e.g., propiconazole and tebuconazole) on germination of *B. cinerea* conidia was strongly enhanced by the presence of sub-lethal doses of some inhibitors of ABC transporters. Another possibility is the use of biocontrol strains which overexpress selected ABC transporters for possible use in integrated pest management in combination with sub-lethal doses of toxicants. These strains may also display enhanced ecological fitness and biocontrol activity in environments characterized by intensive cultivation.

Introduction

ATP-binding cassette (ABC) transporters constitute a large superfamily of membrane-anchored permeases present in a number of evolutionarily distant organisms, from bacteria to human (Higgins 1992). The basic unit of an ABC transporter is composed of (i) a conserved sequence of about 200 amino acid residues which is responsible for ATP hydrolysis and known as ATP-binding cassette, and (ii) of a moiety containing six stretches of hydrophobic amino acid residues which are supposed to be transmembrane domains. In general, a typical ABC transporter is made of two of such units. The main function of ABC permeases involves the transport of a number of substrates coupled to ATP hydrolysis. Ion channel activity has also been described. ABC transporters mediate an increasing number of important biological roles including the protection of cells against accumulation of toxic compounds (Leppert et al. 1990), the secretion of endogenous antibiotics (Buche et al. 1997), steroid hormones (Kralli et al. 1995) and mating factors (McGrath and Varshavski 1989), the uptake of nutrients (Walshaw and Poole 1996), and the clearance of dead cells during differentiation processes (Luciani and Chimini 1996). Impairment of the function of some ABC transporters causes a number of important metabolic disorders in humans, including cystic fibrosis, adrenoleukodystrophy, and Zellweger syndrome. The best characterized human ABC transporters are those conferring, on overexpression, simultaneous resistance to chemically unrelated compounds, a phenomenon known as multidrug resistance (MDR). The mechanism of MDR relies on increased energy-dependent efflux activity of toxic drugs mediated by ABC permeases. MDR is, at the moment, the major obstacle in cancer chemotherapy (Gottesman and Pastan 1993). Disruption of the genes which confer MDR upon overexpression causes increased sensitivity to toxic drugs and metabolic disorders (Smit et al. 1993).

Sequencing of the whole yeast genome predicts, on the basis of homologies, the presence of 29 putative members of the ABC transporters superfamily, whose functions are not yet fully understood (Decottignies and Goffeau 1997). One of them, PDR5, confers resistance to numerous toxins, including triazole fungicides, upon overexpression, whereas disruption of the gene encoding PDR5 causes hypersensitivity to the same compounds (Leppert et al. 1990). The phenomenon of MDR based on energy-dependent increased efflux activity of the toxins has also been described in several species of filamentous fungi. In some cases, pleiotropic effects, such as cold sensitivity have been found to be associated with the occurrence of MDR. A series of loci, including at least ten in *Aspergillus nidulans* and nine in *Neurospora heamatococca*, have been identified as responsible for MDR (De Waard et al. 1996).

On the basis of several genetic and biochemical observations we hypothesized that transport mediated by ABC permeases of filamentous fungi can play a role in a number of processes including: (i) secretion of endogenously produced compounds which influence the surrounding environment (e.g., antibiotics), and (ii) protection from exogenous toxic compounds, including synthetic fungicides and antibiotics present in natural micro-environments. In plant pathogenic fungi ABC permeases may also transport the toxins and enzymes which are necessary for the establishment of a successful parasitic relationship and may defend the fungus from accumulation to toxic levels of plant defense products, which are produced ex novo or released upon colonization attempt.

Materials and Methods

Full length cDNA synthesis of atrB and construction of pYatrB are described in Del Sorbo et al. (1997). Yeast strains JG436 and RW2802 and plasmid pYEura3 were kindly provided by Professor A. Goffeau (Unité de Biochimie Physiologique, Université Catholique de Louvain, Louvain-la-Neuve, Belgium). Yeast drug sensitivity tests were performed as described by Prasad et al. (1995). The effect of co-administration of tebuconazole or propiconazole with inhibitors of ABC transporters on germination of *B. cinerea* was tested by incubating conidial suspensions (10^5/ml) in Czapek medium diluted 1 : 1 with 20 mM Tris-HCl buffer pH 6.0 for 6 to 7 hours with occasional agitation in presence of the drugs, added as ethanol stock solutions (10 mg/ml). Final concentration of ethanol was in all cases 1% (v/v). The doses of ABC transporters inhibitors in absence of the fungicide caused germination inhibition < 5%.

Results and Discussion

We started search for genes encoding ABC transporters in the saprophytic fungus *A. nidulans* and the plant pathogen *Botrytis cinerea.* Using fragments derived from the most conserved regions of *PDR5* we cloned two genes (*atrA* and *atrB*) encoding ABC transporters in the saprophytic fungus *A. nidulans* (Del Sorbo et al. 1997) and one (*bc-atrA*) in the plant pathogen *B. cinerea* (Del Sorbo et al. 1996). The encoded product of all three genes display a high level of homology with yeast PDR5 and display the same topology as PDR5p with respect to their hydropathy profile.

All three genes are constitutively expressed at a low level. Transcription is strongly enhanced following treatment with typical substrates of ABC transporters, such as cycloheximide. For the gene *atrB* enhancement in transcription has also been observed with some triazole fungicides (i.e., imazalil and fenarimol) and with the plant phytoalexin pisatin, indicating that all these substances are potential substrates for AtrBp. Induction takes place within a few minutes after exposure and coincides with the beginning of efflux activity.

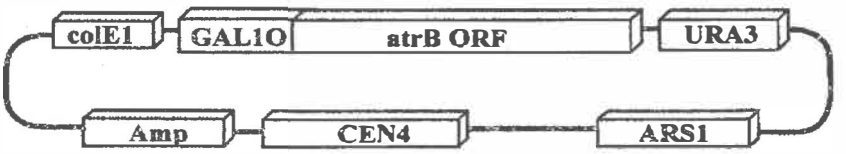

Fig. 1. The shuttle plasmid pYatrB, derivate from pYEura3 and used for complementation of the yeast gene *PDR5*. The genes *colE1* and *Amp* allow propagation of the plasmid in *E. coli* and ampicillin resistance, respectively. *ARS1* and *CEN4* allow autonomous plasmid replication and confer single or low copy number in yeast. *URA3* is the selectable marker and complements uracil auxotrophy. The coding region of the gene *atrB* of *A. nidulans* (atrB ORF) is fused to the promoter region of the galactose-inducible *GAL10* gene.

As a model to study the role of *atrB* in transport of the mentioned compounds, we synthesized its full length cDNA and put it under the control of a galactose-inducible (*GAL10*) promoter of yeast. The construct (pYatrB) (Fig. 1) was then transformed to a yeast strain (JG436) disrupted in *PDR5* and, therefore, displaying hypersensitivity to several antibiotics.

We could observe that, upon induction, the functional expression of *atrB* could restore the resistance to cycloheximide, chloramphenicol, rhodamine 6G and imazalil. This demonstrates that AtrBp of *A. nidulans* is a functional analogue of yeast PDR5p. Interestingly, the expression of *atrB* could totally restore the wild-type level of sensitivity towards some drugs, whereas, for other compounds the level of sensitivity conferred by *atrB* was lower (e.g., chloramphenicol). This indicates a certain degree of specificity towards triazole fungicides of AtrB with respect to PDR5 (Table 1). The role of the other two PDR5p homologues, AtrAp and Bc-atrAp, is still under investigation.

Table 1. Effect of transformation of plasmid pYatrB containing the full-length cDNA of *atrB* in a *PDR5*-disrupted drug hypersensitive mutant of *Saccharomyces cerevisiae* (JG436, mat **a**, *PDR5::Tn5*, *leu2*, *met5*, *ura3-52*, *mak71*, *KRB1*; Leppert et al., 1990).

	Minimal inhibitory concentration (µg/ml)		
Compound	JG436 + pYEura3	RW2802 + pYEura3	JG436 + pYatrB
Cycloheximide	0.03	0.3	0.3
Rhodamine 6G	1.0	10.0	10.0
Chloramphenicol	100.0	>1000.0	300.0
Imazalil	10.0	30.0	30.0

Sensitivity of the disrupted mutant JG436 transformed with the plasmid pYEura3 (i.e., pYatrB without the cDNA of *atrB*, JG436+pYEura3) was used as control. RW2802 is the parental strain of JG436 with a functional copy of *PDR5*.

The possibility that AtrB can transport compounds involved in the maintenance of biological equilibria in natural microenvironments is currently being studied by testing the sensitivity of yeast *PDR5*-deficient mutants complemented with a functional *atrB* cDNA to a number of other substances, especially antibiotics produced by the biocontrol fungi *Trichoderma* and *Gliocladium*. Furthermore, in order to study possible direct interactions of the mentioned substances with the transporter AtrBp, a fluorimetric competitive assay in presence of rhodamine 6G in isogenic yeast strains containing or not AtrB is being developed. Mutants of *A. nidulans* disrupted in *atrA* and/or *atrB* are being generated to study the phenotypic changes in antibiotic and fungicide sensitivity and in fitness associated with impairment of transport function.

Elucidation of the role of ABC transporters in ecology can open new perspectives in biological control strategies. For example, combined administration of synthetic or natural fungicides with selected non-toxic inhibitors of ABC permeases, could allow a substantial reduction in the doses of chemicals employed for control of plant diseases. This strategy is currently being studied by testing the occurrence of synergism between non-lethal dosages of some natural and synthetic inhibitors of ABC transporters and some commercial fungicides on germination inhibition of *B. cinerea* conidia. We observed that the effect of some triazole fungicides, namely propiconazole and tebuconazole, was strongly enhanced in the presence of sub-lethal doses of some known ABC permeases inhibitors, namely *bis*-benzimide, rhodamine 6G, N-ethylmaleimide, and quercetine (Figs. 2 and 3). The synergistic effect was not observed with other transport inhibitors such as daunomycin and quinidine. Currently, the synergistic effect is being tested with some natural antibiotics produced by biocontrol fungi (e.g.,

gliotoxin, trichorzianins), fungal cell wall lytic enzymes (e.g., chitinases and glucanases) and other antibiotics affecting membrane functionality (gramicidin, oligomycin).

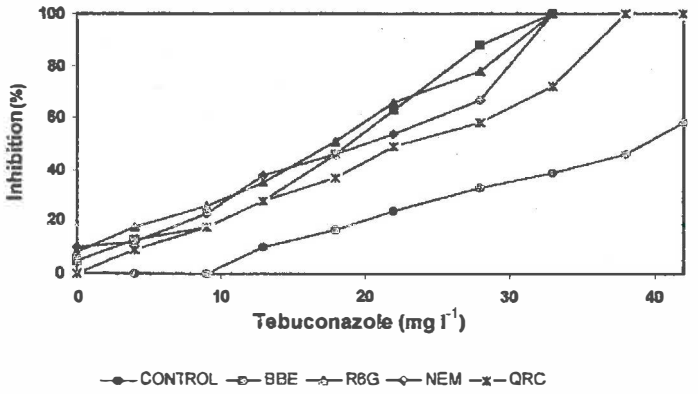

Fig. 2. Effect of co-administration of tebuconazole with sub-lethal doses of some ABC transporters inhibitors (BBE = *bis*-benzimide, 0.6 mg/liter; R6G = rhodamine6G, 0.2 mg/liter; NEM = N-ethyl-maleimide 1 mg/liter; QRC = quercetine, 6 mg/liter) on germination of conidia of *B. cinerea*. Values are means of three independent assays, each performed in triplicate.

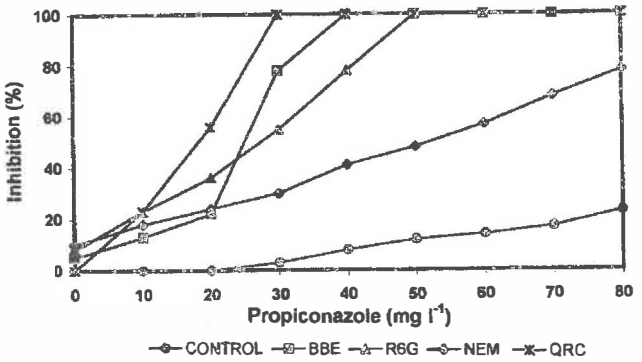

Fig. 3. Effect of co-administration of propiconazole with sublethal doses of some ABC transporters inhibitors on germination of conidia of *B. cinerea*. Abbreviations were as described in the caption to Fig. 2.

Another fascinating possibility is the modulation of ABC permease activity in biocontrol organisms. The overexpression of selected endogenous or artificially introduced ABC permeases could improve biocontrol activity in applications where tolerance to synthetic fungicides or adverse environmental conditions are required, or by increasing the production of antibiotics involved in biocontrol.

New possibilities in biological plant disease control could be opened by direct or indirect interference with ABC-mediated transport processes occurring during pre-penetration and penetration stages. This strategy requires the elucidation of the role of ABC transporters during plant pathogenesis. To gain knowledge in this field, we are currently trying to generate mutants of *B. cinerea* disrupted in the gene *bc-atrA* and studying the pathogenicity and other relevant phenotypic characters related to pathogenicity, such as lytic enzyme production and resistance to plant defense products.

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Molecular approaches in the development of biocontrol agents of foliar and floral bacterial pathogens

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Abstract

Bacterial speck, caused by *Pseudomonas syringae* pv. *tomato*, and bacterial spot, caused by *Xanthomonas axonopodis* pv. *vesicatoria*, both cause economically significant losses in commercial production of fresh-market tomato. Copper bactericides have become ineffective for the control of these diseases in some production areas due to the appearance of copper resistance in the populations of *P. syringae* pv. *tomato* and *X. axonopodis* pv. *vesicatoria*. Furthermore, there is no resistance to either pathogen in commercially available fresh-market tomato varieties. Naturally-occurring non-pathogenic *P. syringae* strain Cit7, originally selected for pre-emptive biocontrol of Ice$^+$ *P. syringae* strains on potato and strawberry, has provided significant reductions in bacterial speck severity in the field at several locations in North America. A nonpathogenic *hrpS* mutant of *P. syringae* pv. *tomato* DC3000 was also effective in reducing bacterial speck severity caused by the homologous pathogenic parental strain under both greenhouse and field conditions, but was no more effective than the naturally-occurring strain *P. syringae* Cit7. Molecular approaches are being employed to improve the biocontrol efficacy of the naturally-occurring strains. The colonization capacity and biocontrol efficacy of *P. syringae* Cit7 against bacterial speck were improved by introduction of the plasmid pNAH7, which confers ability to catabolize salicylate, and exogenous provision of the selective carbon source salicylate. We are in the process of transforming *P. syringae* Cit7 to utilize mannopine and agropine and transforming tomato to produce those same compounds in order to investigate the improvement in colonization capacity and biocontrol efficacy afforded by the ability of the biocontrol agent to catabolize selective carbon and nitrogen sources provided endogenously by a transgenic host. In general, naturally-occurring nonpathogenic bacteria were relatively ineffective for control of bacterial spot. Under greenhouse conditions, however, nonpathogenic *hrp* mutants of *X. axonopodis* pv. *vesicatoria* provided high levels of biocontrol of bacterial spot caused by the homologous pathogenic parental strain. Regulatory *hrpXv* and *hrpG* mutants provided higher levels of biocontrol than the structural *hrpB*, *hrpE*, and *hrpF* mutants. We are currently using molecular tools to investigate the factors involved in the differential efficacy of naturally-occurring strains and the regulatory and structural *hrp* mutants of *X. axonopodis* pv. *vesicatoria* in the biocontrol of bacterial spot.

Introduction

Bacterial pathogens cause a number of economically significant diseases of aerial plant tissues in temperate and tropical climates. Some of the most well known foliar diseases in temperate regions are fire blight of apple and pear, caused by *Erwinia amylovora*, bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, and bacterial spot of tomato, caused by *Xanthomonas axonopodis* pv. *vesicatoria*. In temperate regions, ice-nucleation-active (Ice$^+$) *P. syringae* strains are also incitants of frost injury on sensitive plant tissues. In tropical

regions, bacterial blight of rice, caused by *X. oryzae* pv. *oryzae*, is perhaps one of the most serious foliar bacterial diseases.

Control of these bacterial diseases has traditionally relied upon the use of 'clean', pathogen-free, seed or other planting material; host resistance; appropriate cropping practices; and chemical bactericides. The introduction of resistance genes into crops has contributed greatly to production in areas where bacterial diseases are prevalent. There are, however, limitations to this disease-management strategy: (i) resistance genes are not available for many pathogens in commercial cultivars; (ii) the complex race-structure of bacterial pathogens necessitates appropriate deployment of resistance genes; and further, (iii) resistance genes may be overcome by the pathogen population. Chemical control of bacterial diseases has relied heavily upon the use of copper derivatives and also antibiotics, such as streptomycin, oxytetracycline, and kasugamycin. Unfortunately, copper-resistance and streptomycin-resistance have become prevalent in the populations of several bacterial pathogens, prompting concerns about reduction or loss of efficacy of these bactericides and antibiotics. A new chemical CGA-245704, marketed as Actigard™ in Europe by Novartis Crop Protection, which induces systemic acquired resistance (SAR) in the host plant, has recently been demonstrated to control bacterial speck and spot of tomato. Although chemicals such as this, which activate host-SAR, may be widely used for bacterial disease control in the future, at the present time there is an urgent necessity for alternative control practices for foliar and floral bacterial diseases.

Biological control of bacterial pathogens

Biological control of frost injury. Frost injury of susceptible crops can be incited by Ice^+ strains of *P. syringae* and other leaf-associated bacteria. Injury can be reduced by practices which reduce the population size of these bacteria, since incidence of frost damage is proportional to log population size (Lindow et al. 1982). Lindow (1983) found that prior colonization of a plant by naturally-occurring non-ice-nucleation-active (Ice^-) bacteria reduced subsequent colonization by Ice^+ bacteria. This reduced the incidence of frost injury on those plant tissues. Biological control in which a non-deleterious or non-pathogenic bacterium inoculated onto the plant prevents subsequent colonization by a deleterious or pathogenic bacterium is referred to as pre-emptive competitive exclusion. While naturally-occurring Ice^- strains have been used to provide control of frost injury on crops as diverse as almonds, strawberries, potatoes, and pears, Lindow (1987) demonstrated that highly effective pre-emptive competitive exclusion of Ice^+ *P. syringae* strains could be achieved through the use of engineered Ice^- deletion mutants of the parental wild-type Ice^+ *P. syringae* strains. This was probably the first example of the creation of an innocuous bacterium for use in pre-emptive biocontrol by the removal of a deleterious phenotype through the deletion of a specific region of the genome.

The regulatory climate and prevailing industry and consumer opinion at the time of development of Ice^- *P. syringae* precluded its use as a commercial biocontrol agent of frost injury. However, a naturally-occurring strain, *Pseudomonas fluorescens* A506, was developed for this purpose under the name FrostBan B™. Currently marketed under the name BlightBan™ A506 (Plant Health Technologies, Boise, Idaho, USA), it is registered by the U.S. Environmental Protection Agency for biological control of frost injury on a number of crops, including pear and tomato. In some respects, *P. fluorescens* A506 may be a superior pre-emptive biocontrol agent to engineered Ice^- *P. syringae*, since *P. fluorescens* A506 is

nutritionally very versatile (i.e., it has a broad nutritional niche) compared to *P. syringae*. Hence, *P. fluorescens* A506 is potentially capable of excluding a nutritionally diverse community of Ice$^+$ bacteria, including both *P. syringae* and other species (Wilson and Lindow 1994).

Biological control of fire blight. Fire blight of pear, apple, and rosaceous ornamentals, caused by *E. amylovora*, has become a worldwide problem as the pathogen has spread from North America to Europe, Australia and New Zealand. During frost control studies with *P. fluorescens* A506 in pear it was discovered that this biocontrol agent was also effective against fire blight (Lindow et al. 1996). BlightBan™ A506 is now used in commercial pear production on the west coast of the US in an integrated program with streptomycin antibiotic, to which the organism has natural resistance (Stockwell et al. 1996).

Perhaps because of the success of such naturally-occurring strains, the majority of molecular studies in this area have addressed the mechanisms of action of these biocontrol agents (Vanneste et al. 1992). These studies may in the future lead to engineered derivatives of currently used biocontrol agents with enhanced antibiotic production or survival capabilities, or may lead to improved compatibility between biocontrol agents, such as *P. fluorescens* A506 and another biocontrol agent, *Pantoea agglomerans* C9-1.

Pre-emptive biocontrol of *E. amylovora* infection using spontaneous and Tn5 derived non-pathogenic strains has demonstrated some potential (Bellemann and Geider 1992, Tharaud et al. 1993, 1996). To date, these studies have been conducted only in the growth chamber and greenhouse, partly because of the strict regulations relating to the release of engineered organisms in Europe. One might predict, however, that the nonpathogenic mutants would be no more effective than the naturally-occurring strains in the pre-emptive exclusion of *E. amylovora* from the stigma and nectary and the biocontrol of the blossom-blight phase of the disease. Conceivably non-pathogenic mutants of *E. amylovora* may be able to invade host tissues to a limited extent without causing disease symptoms and induce host-defense responses which may protect against subsequent infection by pathogenic *E. amylovora* (Bellemann und Geider 1992). This would be particularly beneficial in the shoot-blight phase of the disease.

Biological control of bacterial speck of tomato. In a relationship similar to that observed with Ice$^+$ *P. syringae*, the incidence and severity of brown spot of bean, caused by *P. syringae* pv. *syringae*, can be predicted from the leaf surface population size of *P. syringae* pv. *syringae* on bean leaflets at a previous point in time (Rouse et al. 1985). This relationship between surface population size and disease incidence and severity, which probably exists with most *P. syringae* pathovars, predicts that incidence and severity of diseases caused by *P. syringae* pathovars can be reduced by pre-emptive competitive exclusion of the leaf surface population.

We are using biocontrol of bacterial speck of tomato, caused by *P. syringae* pv. *tomato* as a model system in which to examine pre-emptive competitive exclusion as a disease control mechanism and to investigate approaches to enhance the efficacy of foliar biocontrol agents of bacterial diseases. Bacterial speck is a problem in both fresh-market and processing tomato production during cool, wet periods, reducing yields through infection of the leaves and specking of the fruit. While the *Pto* resistance gene has been incorporated into some processing tomato varieties, race 1 of *P. syringae* pv. *tomato* does not contain the *avrPto* gene and, therefore, is still virulent on these lines. Further, copper-resistance has been documented in the *P. syringae* pv. *tomato* population; hence, a biocontrol agent of bacterial speck would be desirable at this time.

In empirical greenhouse assays and field trials we have demonstrated that effective biological control of bacterial speck of tomato can be achieved through prior inoculation of plants with naturally-occurring non-pathogenic bacteria isolated from non-symptomatic tomato leaves and also with strains isolated originally for the pre-emptive exclusion of Ice$^+$ *P. syringae*. In fact, *P. fluorescens* A506, the organism developed for fire blight control as BlightBan^{TM} A506, provided significant reductions in bacterial speck intensity at several locations in North America during field trials in 1996 and 1997 (Campbell et al. 1997, Wilson et al. 1996). Even the non-pathogenic, Ice$^+$ *P. syringae* strains TLP2 and Cit7 can be used as pre-emptive biocontrol agents of *P. syringae* pv. *tomato* (Campbell et al. 1997, Wilson et al. 1996). In fact, Ice$^+$ *P. syringae* Cit7 is consistently more effective than *P. fluorescens* A506 in the control of bacterial speck of tomato (Campbell et al. 1997, Wilson et al. 1996).

Some of our studies have focused on the role of pre-emptive utilization of nutritional resources required for growth of the pathogen by the biocontrol agent (Ji et al. 1997, Wilson and Lindow 1994a, 1994b). Investigations of resource limitation in epiphytic populations of Ice$^+$ *P. syringae* indicated that these populations are primarily limited by carbon availability and secondarily limited by nitrogen availability, and further, that any one of a number of substitutable carbon or nitrogen sources can alleviate the limitation (Wilson and Lindow 1994a, 1994b). In vitro carbon source utilization profiles were determined to provide a reasonable estimate of the in planta nutritional niche of Ice$^+$ *P. syringae* strains and overlap in the nutritional niche of two organisms was quantified using a niche overlap index (NOI). In a random collection of non-pathogenic bacteria isolated from tomato leaves, niche overlap (based on carbon sources reported to be present in tomato leaves) between the non-pathogenic bacterium and the pathogen, *P. syringae* pv. *tomato*, was predictive of biocontrol efficacy (Ji et al. 1997). This suggests, that one important component of pre-emptive biocontrol efficacy is the prior utilization of carbon sources in tomato leaf leachates which are required for growth of the pathogen. This relationship has been further investigated through the creation of catabolic mutants of the biocontrol agent non-pathogenic *P. syringae* strain TLP2 using Tn*5* mutagenesis (Ji et al. 1996). The catabolic mutants, which are altered in ability to catabolize one or more carbon sources in vitro, are reduced in leaf surface population size and reduced in pre-emptive biocontrol efficacy in proportion to their proportional reduction in total carbon source utilization compared to the wild-type parental strain. These data suggest that similarity in carbon source utilization between the putative biocontrol agent and the pathogen is an important, though by no means the sole, prerequisite for successful pre-emptive biocontrol.

Under the assumption that identity of carbon source utilization between biocontrol agent and target pathogen would lead to optimal pre-emptive biocontrol, we are examining the efficacy of hypersensitive response and pathogenicity (*hrp*) mutants of *P. syringae* pv. *tomato* as pre-emptive biocontrol agents of bacterial speck. Perhaps surprisingly, non-pathogenic *hrpA*, *hrpH* (*hrcC*), and *hrpS* mutants of *P. syringae* pv. *tomato* strain DC3000 (Roine et al. 1997) were no more effective as pre-emptive biocontrol agents of the homologous parental strain and bacterial speck disease than *P. syringae* TLP2 or Cit7, under either greenhouse or field conditions. The relative efficacy of these strains needs to be confirmed with additional experiments, but one might speculate that poor leaf surface survival of the *hrp* mutants may at least in part account for the lower efficacy.

Enhancement of biocontrol efficacy in the control of bacterial speck of tomato. Since the phyllosphere of nitrogen-sufficient tomato plants appears to be a carbon-limited environment (Wilson and Lindow 1994a, 1994b), we are examining ways to enhance the population size of biocontrol agents of bacterial speck through provision of selectively

catabolized carbon sources. Such studies also provide a test of the assumption that larger population sizes of a biocontrol agent will result in higher levels of disease control. Two approaches have been adopted, in the first, the carbon source is applied exogenously in conjunction with the biocontrol agent engineered to catabolize it. In the second, the crop plant itself is engineered to exude the selective substrate onto the surface at the sites of colonization of the biocontrol agent engineered to catabolize this substrate. Since we would not necessarily expect a nutritionally-enhanced population of a biocontrol agent to provide superior pre-emptive nutrient utilization and pathogen exclusion, we selected *P. syringae* Cit7 for these studies, because it is our most effective biocontrol agent of bacterial speck and because it may possess biocontrol mechanisms other than pre-emptive carbon utilization.

The plasmid pNAH7 from *P. putida* PpG7, which confers the ability to catabolize salicylate as a sole carbon source, was introduced into the biocontrol agent *P. syringae* Cit7. The transconjugant Cit7(pNAH7) grew on salicylate as the sole carbon source in vitro achieved significantly higher population sizes on tomato leaves amended with salicylate and provided significantly higher levels of biocontrol of bacterial speck on plants amended with salicylate in the greenhouse than the parental strain Cit7 (Ji and Wilson 1997). These studies suggest that, in addition to the SAR effect of salicylate on tomato, the ability of the transconjugant to catabolize an exogenously applied carbon source resulted in higher population sizes and higher levels of biocontrol of bacterial speck. While exogenous salicylate application to support colonization by a salicylate-catabolizing biocontrol agent was effective in a greenhouse system, the necessity to frequently apply an exogenous, water-soluble nutrient source under field conditions would not be practical. Hence, we have generated transgenic tomato plants which produce the selective substrate themselves.

Opines are a unique class of compounds produced by plant cells in galls caused by infection of susceptible plants by *Agrobacterium tumefaciens*. These opines are believed to leach from the gall tissues into the surrounding soil where they provide a selective source of carbon and nitrogen for free-living *A. tumefaciens* cells. The ability to catabolize the mannityl opines mannopine and agropine was conferred upon the biocontrol agent *P. syringae* Cit7 by introduction of the plasmid pYDH208, containing opine catabolic genes from *A. tumefaciens* 15955 (Wilson et al. 1995). On transgenic tobacco (*Nicotiana tabacum* cv. Xanthi, line 2-26) the opine-catabolizing strain Cit7(pYDH208) grew to significantly higher population sizes than the non-opine-catabolizing strain Cit7::*xylE* (Wilson et al. 1995). Transgenic mannityl opine-producing tomato plants have been generated (Wang et al. 1998) and the mannityl opine catabolic genes introduced into the biocontrol agent *P. syringae* Cit7 in a stable pVSP61 derivative (Wang and Wilson *unpublished data*), in order to test the hypothesis, that enhanced population sizes of opine-catabolizing Cit7Mop/Agr on transgenic tomato will provide significantly increased biocontrol of bacterial speck compared to the non-opine-catabolizing parental strain Cit7.

In future, the opine catabolic genes will be cloned into the chromosomal *ice* locus of *P. syringae* Cit7. The *P. syringae* Cit7 *ice* locus has been selected for the future introduction of opine catabolic genes, since previous studies have demonstrated that Ice^- deletion derivatives of Ice^+ Cit7 are as fit as the parent under field conditions. The presence of a 'fitness-neutral' site in the genome of this biocontrol agent permits the introduction of various genes which may contribute to the survival or efficacy of this biocontrol agent. Example include copper resistance genes for integration of this biocontrol agent with copper bactericides in production agriculture (Rogers et al. 1994), ultraviolet radiation resistance genes for improved epiphytic survival (Sundin 1996), antibiotic-production genes targeted

against bacterial or fungal pathogens, or genes for the production of substances, such as harpin, which induce SAR in the host plant. The latter possibility is particularly exciting, since induction of plants by harpin has been reported to provide resistance against several bacterial pathogens. The *hrpN* gene of *E. amylovora* (Wei et al. 1992) or the *hrpZ* gene of *P. syringae* (He et al. 1993) could be cloned directly into the chromosomal *ice* locus behind a suitable promoter. Harpin would likely be exported through the *P. syringae* type III secretion apparatus. Production of harpin by Cit7 on the surface of tomato should provide synergistic levels of speck control due to induction of SAR as well as the other intrinsic mechanisms of Cit7.

Biological control of bacterial spot of tomato. Bacterial spot of tomato and pepper is caused by *X. axonopodis* pv *vesicatoria*. While host resistance genes are available in commercial varieties of pepper, host resistance has not yet been incorporated into commercial tomato varieties. Even in pepper, the complex race structure of the pathogen and variable geographic distribution of the different races means that multiple resistance genes have to be incorporated into new varieties and that the resistance genes have to be deployed appropriately depending on the location. To add to these problems, copper resistance is now widespread in the *X. axonopodis* pv. *vesicatoria* population.

For pre-emptive biocontrol of foliar bacterial diseases to be possible, there must be a relationship between disease incidence or severity and the size of the leaf surface pathogen population; otherwise, pre-emptive exclusion of the leaf surface pathogen population will not result in reductions in disease. While *X. axonopodis* pv. *vesicatoria* has been reported on several occasions to multiply on the leaf surface in the absence of symptoms, such a relationship has not been demonstrated and in fact, we have preliminary evidence that this relationship does not occur in *X. axonopodis* pv. *vesicatoria*. Initially it was assumed that the use of naturally-occurring strains to reduce the leaf surface population size would result in disease control. The first observation which suggested that this may not be the case was that many fewer strains resulted in pre-emptive biocontrol of spot than speck, while the second was that levels of disease reduction in the field, although consistent, were much lower with spot than speck (Byrne et al. 1998). Subsequent analysis of population reduction and greenhouse disease severity reduction data indicated that there was no relationship between these two variables (Dianese and Wilson 1996). We now believe that these observations point to a fundamental difference in biology between *P. syringae* and *X. campestris*. The populations of *X. axonopodis* pv. *vesicatoria* derived from leaf washings may represent surface populations multiplying after and independent of internal invasion, or may represent cells washed out of substomatal cavities or incipient lesions. This situation necessitates different biocontrol strategies for *X. axonopodis* pv. *vesicatoria* concentrating on preventing infection and reducing internal multiplication.

Certain non-pathogenic *hrp* mutants of *X. axonopodis* pv. *vesicatoria* have proven to be highly effective biocontrol agents of bacterial spot under greenhouse and field conditions. Inoculation of plants with these *hrp* mutants 24 h prior to inoculation with the wild-type pathogenic parent has no effect on either internal or external leaf colonization by the pathogenic parent, but disease levels are significantly reduced. Interestingly, *hrp* mutants with mutations in the regulatory loci *hrpXv* and *hrpG* are significantly more effective in the control of spot than the *hrp* mutants with mutations in the structural loci *hrpB*, *hrpE*, and *hrpF* (Moss et al. 1997, 1998). A currently uncharacterized mutant, which multiplies on the plant surface and interior but which is apparently non-pathogenic is even more effective than the *hrpXv* and *hrpG* mutants (Moss et al. 1997). This mutant may turn out to be analogous to the lesion

minus *lem*-mutants of *P. syringae* (Willis et al. 1990) or the more recently described disease specific (*dsp*) mutants (Bellemann and Geider 1992). We are currently examining the mechanisms of action of these non-pathogenic mutants, which apparently do not include pre-emptive utilization of nutritional resources and exclusion of the parental strain, but may relate to differential induction or suppression of host defense responses.

We are also interested in developing other novel approaches for the prevention of infection by *X. axonopodis* pv. *vesicatoria*. One such approach could involve external interference with pathogen cell-cell signaling involved in density-dependent regulation of phenotypes related to pathogenesis. Several Gram-negative phytopathogenic bacteria, includeing *A. tumefaciens* (Hwang et al. 1994), *Erwinia carotovora* (Pirhonen et al. 1993), *Erwinia stewartii* (Von Bodman and Farrand 1995), exhibit density-dependent gene regulation, mediated through the production of *N*-acyl homoserine lactone (*N*-HSL) autoinducers of the type originally described in *Vibrio fischeri*. In *E. carotovora* exoenzyme production is regulated in a density-dependent fashion, with the result that the onset of infection and soft rot requires a high density of cells and a high concentration of the *N*-HSL autoinducer to accumulate on the plant tissue before genes encoding lytic enzymes involved in cell-wall breakdown are turned on. It is still unclear whether *X. axonopodis* pv. *vesicatoria* or other *X. campestris* pathovars produce *N*-HSLs, however, there is some evidence that *X. campestris* has evolved other cell-cell signaling systems that may be involved in pathogenicity. In *X. campestris* pv. *campestris*, mutations in the gene *rpfF*, characterized by downregulated exoenzyme synthesis and reduced virulence, can be complemented by diffusible extracellular factor produced by most strains of *X. campestris* pv. *campestris* (Barber et al. 1997). In another case, *pigB* mutants of *X. campestris* pv. *campestris*, characterized by altered EPS and xanthomonadin production in vitro and reduced virulence in planta, are restored to full EPS and xanthomonadin in vitro by a small molecular weight diffusible factor, and fitness in leaf surface colonization and virulence are partially complemented in planta by coinoculation with strains producing the diffusible factor (Poplawsky and Chun 1997). Investigations of such cell-cell signaling mechanisms are underway and may reveal ways in which we can interfere with cell-cell communication, perhaps through production of inactive signaling molecules by engineered biocontrol agents, which competitively bind receptors in the pathogen and mitigate transcription of genes involved in pathogenesis.

Biological control of disease cause by other *Xanthomonas* species. From the literature it is unclear whether other *Xanthomonas* species or other *X. campestris* pathovars behave in a similar fashion to *X. axonopodis* pv. *vesicatoria*, or are more similar to *P. syringae* in the relationship between surface populations and disease incidence or severity. One recent study suggested that leaf surface population size of *X. translucens* pv. *translucens* may be related to bacterial leaf streak severity on wheat (Stromberg et al. 1997), however, the majority of investigations have not critically addressed the role of surface populations prior to launching into screening potential biocontrol agents.

We are interested in the application of the biocontrol approaches described to diseases caused by other *Xanthomonas* species and *X. campestris* pathovars. Of particular interest is the potential for development of a biological control system for bacterial blight of rice, caused by *X. oryzae* pv. *oryzae*, to complement current breeding programs which are focusing on appropriate deployment of resistance gene for this pathogen which has a complex race structure and geographic distribution. Some preliminary field data from Nanjing, China (C. He *unpublished data*) suggest that *hrp* mutants of *X. oryzae* pv. *oryzae* provide some control of bacterial blight of rice.

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In vitro compatibility between fluorescent *Pseudomonas* spp. strains can increase effectivity of Fusarium wilt control by combinations of these strains

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Abstract

In vitro interactions between Fusarium wilt suppressing *Pseudomonas* spp. strains RE8 and RS111 were investigated. Growth of strain RS111 on SSM agar medium was strongly inhibited by strain RE8, whereas RE8 was not inhibited by RS111. From the inhibition zone, produced by RE8, a mutant of strain RS111 (RS111-a) was isolated, that was no longer inhibited by RE8. When applied together in soil, the incompatible combination of RE8 plus RS111 did not result in a better suppression of Fusarium wilt of radish as compared to the single strains. However, the compatible combination RE8 plus RS111-a did result in a better disease suppression as compared to the single strains and to the incompatible combination. Thus, better performance in biocontrol by the combination of strains RE8 and RS111 appears to be dependent on in vitro compatibility between the strains.

Introduction

Wilt diseases caused by *Fusarium oxysporum* can lead to significant yield losses of horticultural and agricultural crops. Possibilities to manage Fusarium wilt, such as with fungicides, are limited. Therefore, other strategies to control this disease, including biological control, are being developed. Microorganisms, and especially fluorescent pseudomonads and non-pathogenic strains of *F. oxysporum* isolated from Fusarium wilt suppressive soils, have the ability to reduce Fusarium wilt. Mechanisms demonstrated to be involved in suppression of this disease include (i) competition for substrate, (ii) siderophore-mediated competition for iron, and (iii) induction of systemic resistance. It is postulated that in disease-suppressive soils a concerted action of several disease-suppressing microorganisms and mechanisms is responsible for the highly consistent disease suppressiveness (Alabouvette 1986, Lemanceau and Alabouvette 1991, Schippers 1992). Application of a mixture of different biocontrol agents may more closely mimic the natural situation and be a more viable control strategy (Duffy et al. 1996). Several authors have shown that combining disease-suppressive microorganisms results in improved biocontrol (Duffy et al. 1996, Leeman et al. 1996, Lemanceau et al. 1992, Pierson and Weller 1994).

It has been postulated that an important prerequisite for successful co-inoculation of strains is the compatibility of the co-inoculated microorganisms (Baker 1990, Li and Alexander 1988, Raaijmakers et al. 1995). Thus, incompatibility of the co-inoculants may explain the reports of combinations of biological control agents that do not result in improved suppression of disease as compared to the separate inoculants (Dandurand and Knudsen 1993, Hubbard et al. 1983, Sneh et al. 1984).

The objective of this study was to determine whether interactions between *Pseudomonas* spp. strains influence disease suppression by combinations of these strains. Interactions

between disease-suppressive *Pseudomonas* strains RE8 and RS111 were first studied in vitro. Subsequently, suppression of Fusarium wilt of radish by the single strains and combinations of strains was investigated to determine in how far interactions in vitro are predictive of disease suppression by combinations of these *Pseudomonas* strains in vivo.

Materials and Methods

Pseudomonas putida strain RE8 was isolated from radish root tissue. *Pseudomonas fluorescens* strain RS111 was isolated the rhizosphere of tomato (Van Peer et al. 1990). RS111-a, a mutant of RS111, was isolated from the inhibition zone surrounding a colony of strain RE8. This mutant was not inhibited in vitro by strain RE8. The pathogen used was *F. oxysporum* Schlecht f.sp. *raphani* Kendrick & Snyder, strain WCS600 (Leeman et al. 1996). Compatibility between *P. fluorescens* strains RS111 or RS111-a and *P. putida* strain RE8 was tested in vitro on standard succinate medium (SSM) agar plates (Meyer et al. 1978) without or with $FeCl_3$ (200 mM). Test strains were spot-inoculated. After 48 h of incubation at 27 °C a suspension of the target strain (10^7 CFU/ml) was atomized over the spot-inoculated plates. Zones of growth inhibition of the overlay strains around the spot-inoculated strains were scored after an additional incubation period of 24 h at 27 °C.

To study the relation between in vitro compatibility and disease suppression, single strains and their combinations were tested for suppression of Fusarium wilt of radish in a potting mix composed of potting soil and river sand. The pathogen was cultured in a shaking culture of 2% malt extract (Difco, Detroit, MI) at 22 °C for 14 days. Inoculum of *F. oxysporum* was produced by mixing microconidia into a non-autoclaved potting mix to give a final concentration of 3.75×10^5 microconidia/g mix. The infested potting mix was incubated for 3 to 5 days at 20 °C. Bacteria were grown for 24 h at 27 °C on King's medium B (KB) agar plates (King et al. 1954) and scraped into water. Bacterial suspensions were mixed into twice autoclaved potting mix to give a approximately 7×10^6 CFU/g mix. For the bioassay, potting mix infested with *Fusarium* and bacteria were added to noninoculated mix (soil but not sand was autoclaved) to give 10^4 *Fusarium* microconidia/g and 10^6 CFU bacteria/ml (or 2×10^6 CFU/g for the bacterial combinations. Treatments consisted of nine pots filled with approximately 750 g potting mix. Ten radish seeds (*Raphanus sativus* L.; cultivar Saxa 2*Nova, S&G Seeds B.V. Enkhuizen) were planted per pot. Plants were grown in the greenhouse at 20 °C with a 16 h photoperiod. After approximately 21 days, the percentage of diseased plants was scored (Leeman et al. 1996). Results of two bioassays were pooled after establishing that there was no significant interaction ($P = 0.05$) between experiments and treatments, and that variances were homogeneous. The pooled data were analyzed for significance with ANOVA followed by Fisher's LSD test ($P = 0.05$) using SAS-software (SAS Institute, Cary, NC).

Results

Pseudomonas fluorescens strain RS111 was strongly inhibited in vitro by strain RE8 (Table 1) on SSM agar plates without or with $FeCl_3$, whereas, strain RE8 was not inhibited by RS111 (results not shown). Mutant RS111-a was less sensitive to inhibition by RE8 on plates without Fe and was not inhibited on plates with Fe (Table 1). Thus, an incompatible (RE8 plus RS111) and a compatible (RE8 plus RS111-a) combination of *Pseudomonas* spp. strains was established. Disease suppression by the incompatible combination of RE8 and RS111 was

comparable to the effects of the single strains. However, disease suppression by the compatible combination of RE8 and RS111-a was significantly better as compared to the single strains (Table 2).

Table 1. In vitro antagonism of *Pseudomonas putida* strain RE8 against *P. fluorescens* strain RS111 and mutant RS111-a on SSM agar plates without or with $FeCl_3$ (200 mM).

Overlay strain	SSM	SSM + Fe
RS111	+ 5.2[z]	+ 2.2
RS111-a	± 3.3	-

[z] '-' indicates no growth inhibition of the overlay strain around the spot-inoculated colony, '±' indicates slight growth inhibition, '+' indicates strong growth inhibition. Values represent the diameter of inhibition zone/colony diameter of RE8.

Table 2. Effect of bacteria used alone and in combination on Fusarium wilt of radish.

Treatment[y]	% Diseased plants[z]
Control	62.2 a
RE8	38.2 b
RS111	40.9 b
RS111-1	37.4 b
RE8 plus RS111	39.4 b
RE8 plus RS111-a	28.3 c

[y] Mix was bacterized with fluorescent *Pseudomonas* spp. strains RE8, RS111, RS111-a (10^6 CFU/g potting mix) or combinations of RE8 with RS111 or RS111-a (10^6 CFU/g potting mix each). The pathogen was mixed through the potting mix to a final concentration of 10^4 microconidia/g mix. Percentage of diseased plants was scored 21 days after sowing.

[z] Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's LSD test.

Discussion

The introduction of biocontrol microorganisms or combinations of these organisms does not always result in a significant and consistent disease suppression. Numerous biotic and abiotic factors are likely to contribute to this variable performance of biocontrol microorganisms (Weller 1988). Inadequate colonization of the rhizosphere, limited tolerance to changes in environmental conditions, and fluctuating production or activity of antifungal metabolites are among the most important factors (Duffy et al. 1996, Pierson and Weller 1994). Several authors have suggested that combinations of introduced biocontrol agents can act more reliably but strains have to be compatible in order to establish a better and more consistent disease suppression (Baker 1990, Janisiewicz 1996, Janisiewicz and Bors 1995, Raaijmakers et al. 1995).

To achieve a better disease suppression by the combination of *P. putida* strain RE8 and *P. fluorescens* strain RS111, in vitro compatibility seems to be a prerequisite. A probable

explanation for these observations is that interactions between the strains influence root colonization. When RS111 is mixed through soil together with RE8, RS111 is probably inhibited in growth and not able to sufficiently colonize the roots. Mutant RS111-a is not inhibited by RE8 and is apparently not impaired in the colonization of the roots in the presence of RE8. Hence, under these conditions, both RE8 and RS111-a can fully express their disease-suppressive properties resulting in an enhanced disease suppression. These results indicate that interactions between biocontrol strains can influence the disease suppression by the combination of these strains. Therefore, it is essential to investigate microbial interactions that enhance or detract from biocontrol (Handelsman and Stabb 1996) to understand and predict the performance of biocontrol agents and strain combinations.

Currently the population dynamics of the strains RE8, RS111, RS111-a and their combinations are under investigation using the immunofluorescence colony staining technique, as modified by Leeman et al. (1995). The disease-suppressive mechanisms of the strains are being elucidated. Disease suppression by combinations of RS111 or RS111-a with several other *Pseudomonas* spp. strains that exhibit similar in vitro interactions is currently being investigated.

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The role of extracellular polysaccharide and regulated gene expression in invasion and colonization of tomato by *Ralstonia solanacearum*

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Abstract

Expression of some virulence genes increases over 20 fold during exponential growth of *Ralstonia solanacearum* in culture. These cell density-associated changes suggest that autoregulators may control a shift between low and high virulence phenotypes, and we hypothesize that this shift might occur during pathogenesis as the pathogen moves from the soil into plant roots and then colonizes the stem. Therefore, we examined invasion of unwounded tomato roots and systemic colonization by a wild-type strain and two virulence mutants. Besides causing wilt symptoms directly, the acidic extracellular polysaccharide (EPS I) promotes rapid systemic colonization of tomato plants, thus increasing the pathogenic/reproductive fitness of *R. solanacearum*. Endoglucanase is less important for systemic colonization. We discovered that *R. solanacearum* has two separate autoinduction systems that are part of a hierarchical signal cascade. The upstream system uses the novel autoregulator 3-hydroxypalmitic acid methyl ester, and has an essential role in regulating virulence. The downstream autoregulatory system uses acylhomoserine lactone autoinducers similar to those in many gram-negative bacteria, but has no obvious role in regulating virulence. We hope that a better understanding how EPS I promotes colonization and how the regulatory network controls production of EPS I will lead to new strategies for disease control.

Introduction

The ability of *Ralstonia solanacearum* to wilt plants is mainly due to its production of an acidic extracellular polysaccharide (EPS I), which can occlude vessels and prevent water flow (Denny 1995). Extracellular proteins as a whole also have a major role in pathogenesis (Kang et al. 1994), but individual plant cell wall-degrading enzymes [e.g., polygalacturonase (PG) and endoglucanase (EG)] are not essential, because their absence only slows the rate of wilting. Production of these virulence determinants is controlled by a complex regulatory network that responds to environmental signals (Schell 1996; and Fig. 3). Central to this network is PhcA, an unusual LysR-type transcriptional regulator that has global effects. Inactivation of *phcA* greatly reduces production of both EPS I and many extracellular proteins, including some cell wall-degrading enzymes.

Several years ago we discovered that, besides several unknown signals, expression of PhcA-regulated virulence genes in *R. solanacearum* is controlled by an extracellular compound that it synthesizes (Clough et al. 1994). This type of gene regulation is similar to the autoinduction phenomenon observed in numerous other bacteria (Fuqua et al. 1996). Autoinduction, also called quorum sensing, is observed when bacteria within a population

coordinately control expression of selected genes in response to the extracellular concentration of an endogenous autoregulator compound. This observation suggested that *R. solanacearum* may have a very different phenotype when surviving at low cell density in the soil than when multiplying rapidly within host tissues (Denny et al. 1994). If this model is true, then it becomes important to know when and where expression of virulence genes is activated during pathogenesis. Does this occur in the rhizosphere, on the root surface, or within the root cortex or stele? Are the virulence factors important for penetration or colonization of roots or only for inducing wilt symptoms? We consequently initiated an in-depth study of cell density-dependent regulation of virulence genes in *R. solanacearum* and the role of autoregulators in this process. We also reexamined the role of virulence factors in invasion and colonization of tomato plants as a prelude to examining virulence gene regulation during pathogenesis. This chapter will briefly review our most salient findings in these areas.

Differential expression of virulence and motility. To investigate whether transcription of genes that encode EPS I biosynthesis (*eps*) and EG (*egl*) might be cell density-dependent we determined the β-galactosidase activity encoded by *lacZ* reporter fusions in these genes throughout exponential phase and the transition to stationary phase (Clough et al. 1997a). In AW1-130, expression of an *eps::lacZ* fusion was very low prior to cultures reaching 1×10^7 CFU/ml, but β-galactosidase activity per cell increased about 100-fold before the cell density reached 5×10^8 CFU/ml (Fig. 1A). As the cultures exited the exponential phase the β-galactosidase activity per cell decreased, likely due to a combination of decreased gene expression and smaller cell size. Experiments with AW1-28 (*egl::lacZ*) revealed a similar pattern of gene expression.

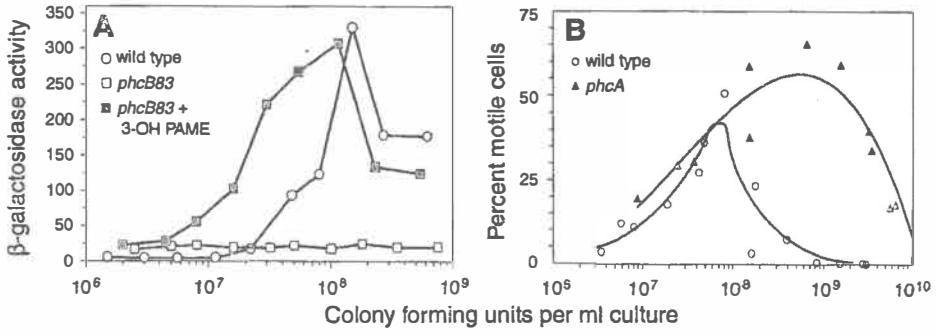

Fig. 1. Expression of *eps* and motility during exponential multiplication of *R. solanacearum*. (A) Expression of *eps::lacZ* in a wild-type strain and a phcB83 mutant in the absence (open symbols) and presence (filled squares) of 35 nM 3-OH PAME added to the culture at time zero. β-galactosidase activity is given as pmol methylumbelliferyl released 10^{-8} cells min^{-1}. (B) Motility of a wild-type strain and a *phcA* mutant; motility of a *phcB* mutant was similar to that for the *phcA* strain.

Cells from colonies of wild-type *R. solanacearum* are essentially nonmotile, whereas comparable cells of *phcA* or *phcB* mutant colonies are highly motile. However, by starting batch cultures at 10^4 CFU/ml and analyzing motility throughout the exponential phase, we found that some of the wild-type cells were transiently motile beginning at about 5×10^6 CFU/ml (Fig. 1B) (Clough et al. 1997a). The fraction of motile cells peaked at 30 to 50% near a cell density of 5×10^7 CFU/ml, and subsequently declined to about 1% at 1×10^9 CFU/ml. The average swimming speed for wild-type cells at 1×10^8 CFU/ml was 29.9 m/sec (SD + 4.1). *Ralstonia solanacearum* strains K60 and GMI1000 also were transiently motile near 10^8 CFU/ml (*data not shown*). The decrease in motility of AW1 at higher densities was not due to increased viscosity resulting from EPS production because AW-19A, an EPS I- strain, was transiently motile similar to the wild-type parent. More importantly, the presence of motile cells in the wild-type culture was not due to the presence of spontaneous *phcA* mutants, because no EPS I- colonies were recovered in the >1,000 CFU plated at each time point. A *phcA* mutant also was transiently motile, but unlike the wild type, 50 to 65% of the cells were still motile at 1×10^9 CFU/ml (Fig. 1B). For this mutant, motility did not decrease until cell densities became greater than 10^9 CFU/ml, presumably due to the gradual exhaustion of the proton motive force during the onset of stationary phase.

These results demonstrated that in culture *R. solanacearum* alternates between two specialized phenotypes and that PhcA has a central role in this process. Above 10^8 CFU/ml, the wild type has what could be considered a high-virulence phenotype. Production of high levels of EPS I and plant cell-wall degrading enzymes associated with this phenotype might be beneficial for colonization of host tissues or evading host defenses. At cell densities below 10^7 CFU/ml the wild type presumably resembles a *phcA* mutant in being low in virulence due to producing low levels of EPS I and certain plant cell-wall degrading enzymes; this phenotype might be well-suited for survival in soil, a saprophytic existence, and/or early parasitic activities.

Role of EPS I in invasion and colonization. Natural infection of tomato roots by *R. solanacearum* occurs either via wounds, the apical elongation zone, or at points of secondary root emergence (Schmit 1978, Vasse et al. 1995). Histologic examination of a susceptible cultivar grown hydroponically revealed that bacteria proliferate within the root cortex near these infection courts within 1 day. Within 2 to 3 days bacteria colonize intercellular spaces of the inner cortex next to the vascular cylinder, and adjacent cells begin to degenerate. At this point, bacteria rapidly infect the stele (central cylinder), appearing in intercellular spaces near the protoxylem, and invade xylem vessels either from ruptured tyloses or by breaching vessel walls.

Since expression of virulence genes is likely to be induced during pathogenesis, we reexamined the role of EPS I and EG in root infection and stem colonization of potted tomato plants by drenching the soil of 4-week old potted tomato plants and then enumerating over time *R. solanacearum* recovered from soil, roots, and stems (Saile et al. 1997). Because soil populations of the wild type and mutants defective in one or both virulence factors did not decrease for >4 days, we concluded that the strains survived equally well during the infection period. At 4 and 24 hours after soil infestation, 60 to 100% of primary roots were infected by each strain and similar CFU/g were recovered from surface-disinfested primary roots, suggesting that neither EPS I nor EG is essential for invasion and initial infection of root tissues.

By sampling the roots and multiple 3-cm stem segments, we found that on days 5 and 12 the EG-mutant had colonized the stems of about half as many plants as the wild type, and the

EPS I-mutants had colonized only 10% (Fig. 2; Saile et al. 1997). In this and two additional experiments, differences between the wild type and EPS I-mutants were statistically significant ($P < 0.05$) using the nonparametric Wilcoxon rank-sum test and by analysis of variance and regression. Similar results were found when the inoculum was applied to the cut end of a leaf petiole (*data not shown*). Thus, we believe that EPS I, and to a lesser extent EG, facilitates rapid systemic colonization of tomato stems by *R. solanacearum*.

It should be noted, however, that EPS I is not the only factor made by *R. solanacearum* that is required for efficient colonization of tomato plants. AW1 *eep* mutants, which are normal for EPS I production but do not export the known plant cell wall-degrading enzymes, are very low in virulence and do not readily move away from the site of inoculation (Kang et al. 1994, Saile *unpublished results*). Strain GMI1000 *hrp* mutants, which have lost pathogenicity on hosts and the ability to elicit the hypersensitive response on nonhosts, invade unwounded roots as readily as the wild-type parent. However, despite producing normal amounts of EPS I, the mutants colonize only the lower parts of the stem and in those regions are present in much lower (10^3 to 10^4 fold less) numbers (Frey et al. 1994). Because *hrp* mutants colonize tomato plants without causing disease, they are being evaluated for use as a biological control agent to prevent subsequent colonization by wild-type strains of *R. solanacearum* (Frey et al. 1994).

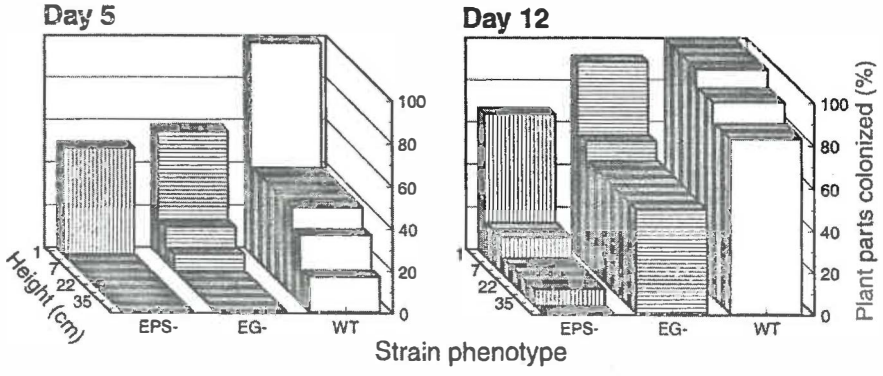

Fig. 2. Colonization of tomato primary roots and stems by *R. solanacearum* wild type or mutants after soil infestation. Multiple 3-cm stem sections were removed from 12 plants for each treatment at 5 and 12 days after infestation. Data are the percentage of sections at each sample height from which viable bacteria were recovered.

3-OH PAME is a novel autoregulator of virulence gene expression. Expression of PhcA-regulated genes requires an extracellular factor that appears in culture supernatant and the air above plate cultures of wild-type *R. solanacearum* (Clough et al. 1994). Production of this volatile extracellular factor requires the *phcB* locus. Some nonpolar mutations in *phcB* result in a phenotype indistinguishable from that of a *phcA* mutant, except that *phcB* mutants can be restored to the wild-type phenotype by addition of the extracellular factor. Gas chromatography-mass spectral analysis (Flavier et al. 1997b) showed that a purified extracellular factor preparation contained a single compound with an ionization profile

matching 3-hydroxypalmitic acid methyl ester (3-OH PAME) (see Fig. 3 for structure). Purified extracellular factor and authentic 3-OH PAME had similar specific activities in restoring *eps* expression in a *phcB* background, and restored wild-type levels of the virulence factors and the cell-density associated expression of *eps* (Fig. 1A). Biologically active concentrations of 3-OH PAME were detected before *eps* expression, suggesting that 3-OH PAME acts as a novel autoregulator in *R. solanacearum*. 3-OH PAME may be the first example of a new family of compounds that can mediate long distance intercellular communication. This possibility is supported by the observation that addition of exogenous 3-OH PAME reduced the cell density at which expression of *eps* is activated. However, exogenous 3-OH PAME did not activated expression immediately after addition to the growing culture (Fig. 1A), which indicates that one or more additional factors co-limit expression of PhcA-regulated virulence genes.

Based on DNA sequencing, mutation studies, and protein synthesis in *Escherichia coli*, we determined that the *phcB* locus contains one operon with four genes (Clough et al. 1997b). The first gene, *phcB*, is essential for production of 3-OH PAME. The presence of a tripartite amino acid motif in PhcB strongly indicates that it is an S-adenosylmethionine-dependent methyltransferase (Flavier et al. 1997b). We hypothesize that PhcB synthesizes 3-OH PAME by displacing the acyl carrier protein (ACP) from 3-OH palmitoyl-ACP, which is a probable intermediate in the production of the fatty acid moiety of lipopolysaccharides in *R. solanacearum*. The other genes in the putative operon are predicted to encode proteins homologous to two-component signal transduction systems; PhcS has amino acid similarity to histidine-kinase sensors, whereas PhcR and PhcQ are similar to response regulators. However, PhcR is quite unusual, because its output domain strongly resembles the histidine-kinase domain of a sensor protein rather than containing the more typical DNA-binding domain. Mutants with nonpolar insertions in *phcB* (i.e., they express *phcSRQ* in the absence of 3-OH PAME) produced 10 to 100-fold reduced amounts of the PhcA-regulated traits EPS I and EG; wild-type phenotype was restored by addition of 3-OH PAME to growing cultures. The genetic data suggest that, in the absence of 3-OH PAME, PhcS and PhcR function together to reduce expression of *phcA*. One model (Fig. 3) consistent with the data is that, at low levels of the 3-OH PAME, PhcS phosphorylates PhcR, which in turn (maybe via an additional, unknown component) reduces expression of *phcA* or the transcriptional activity of PhcA. Once 3-OH PAME reaches a threshold concentration, we suggest that it reduces the ability of PhcS to phosphorylate PhcR, thus leading to increased amounts of functional PhcA.

Acylhomoserine lactone-dependent autoinduction does not control virulence. Autoinduction often involves the synthesis of and response to one or more diffusible acylhomoserine lactones (AHLs) (Fuqua et al. 1996). All of the known AHLs have the same homoserine moiety, but differ in the length and structure of their side chains. Since AHLs are thought to diffuse freely across bacterial membranes, intracellular concentrations that trigger autoinducer-dependent gene expression occur only when a population reaches a sufficient cell density (the necessary quorum) within a confined space. In most bacteria, the enzyme responsible for AHL synthesis is a homologue of *Vibrio fischeri* LuxI; each LuxI homologue synthesizes one or more AHLs. To sense and respond to these autoinducers, most bacteria have a transcriptional activator that is homologous to *V. fischeri* LuxR; generally, LuxR homologues preferentially sense the AHLs made by their cognate LuxI protein.

We determined that most strains of *R. solanacearum* produce one or more AHLs (Flavier et al. 1997a). Strain AW1 makes two AHLs that, based on HPLC analysis, appear to be N-hexanoyl- and N-octanoyl-HSL. Three loci involved in AHL production were identified

using transposon mutagenesis. DNA sequencing of one locus showed that it contains the *luxR* and *luxI* homologues *solR* and *solI*, respectively. Expression of *solI* and *solR* required PhcA, and exhibited a density-associated pattern similar to other PhcA-regulated virulence genes. SolI and SolR are part of a functional autoinduction system in *R. solanacearum*, because a gene (*aidA*, with unknown function) was identified that requires AHLs for expression (see Fig. 3). Inactivation of *solI* abolished production of both autoinducers, but did not affect expression of virulence genes or the ability to wilt tomato. Thus, virulence in *R. solanacearum* does not appear to be regulated by AHL-mediated autoinduction, but because AHL production is part of the PhcA regulon (Fig. 3) it may still have a role in pathogenesis or survival.

A second locus in *R. solanacearum* that affects AHL production contains a gene, $rpoS_{R60}$, encoding a homologue of the RpoS alternative sigma factor Flavier et al. 1998). In other bacteria, RpoS contributes to tolerance of various environmental stresses (Loewen and Hengge-Aronis 1994). A mutation in $rpoS_{R60}$ reduced survival of *R. solanacearum* during starvation and low pH conditions, but did not affect survival in the presence of hydrogen peroxide, or under conditions of high osmolarity or high temperature. Transcription of *solR* and *solI* was decreased more than 10-fold in an $rpoS_{R60}$ mutant background, thereby explaining the reduced AHL production. Although the production of several virulence factors was altered in the $rpoS_{R60}$ mutant, virulence on tomato was only slightly reduced. A mutation in the third locus required for normal AHL production also affects production of several virulence determinants, but this locus has not been genetically characterized.

The *R. solanacearum* regulatory network. A model showing the complex regulatory network of *R. solanacearum* is presented in Fig. 3. For simplicity, only the major, well documented regulatory circuits of this network are shown. PhcA is the primary regulatory component, directly or indirectly affecting production of at least the seven traits shown. PhcA also positively autoregulates transcription of *phcA* (Brumbley et al. 1993) and appears to be necessary for wild-type production of 3-OH PAME (Clough and Denny *unpublished data*). $RpoS_{R60}$ is second in the number of traits that it affects, because it is important for wild-type production of AHLs, EG, and PG, and for survival of acid conditions. The effect of inactivating $rpoS_{R60}$ on PG activity is not shown in Fig. 3, because with only a five-fold change this was considered marginally important. However, since *R. solanacearum* makes three PG enzymes (one endo- and two exo-types) it is possible that production of one of them is more strongly regulated by $RpoS_{R60}$. Also not shown is the contribution of $RpoS_{R60}$ to survival at pH 4, because the mechanism for this tolerance is not yet known. The discovery that $RpoS_{R60}$ is essential for expression of the AHL-dependent autoinduction system in *R. solanacearum* means that this system is controlled by at least two global regulators, and suggests that it has an important role in the bacterium's life cycle. Although only two genes (*solI* and *aidA*) are known to be regulated by the AHL-dependent autoinduction system (Flavier et al. 1997a), because *solR* expression is regulated coincidently with the PhcA-regulated virulence genes, this system is more likely to have a role in planta during pathogenesis.

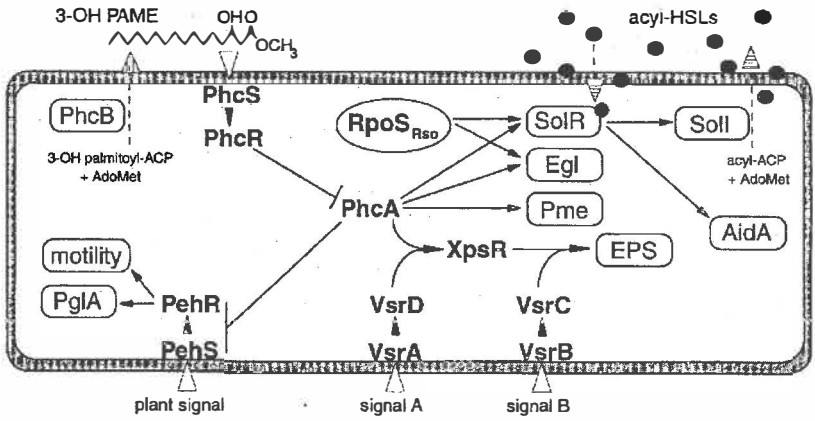

Fig. 3. Model of the regulatory network in *R. solanacearum*. Within this network the four known two-component regulatory systems are PhcS-PhcR, PehS-PehR, VsrA-VsrD, and VsrB-VsrC. Except for 3-OH PAME, the novel autoinducer sensed by PhcS-PhcR, the signals for the two-component systems are not known. Other transcriptional regulators are PhcA (a LysR-type protein) and SolR (a LuxR-like protein that responds to AHLs). Also involved with regulating transcription are $RpoS_{R60}$ (an alternative sigma factor) and XpsR (a signal integrator). Proteins essential for biosynthesis of extracellular signal molecules are PhcB (predicted to be an S-adenosylmethionine-dependent methyltransferase) and SolI (a putative LuxI-like AHL synthase). Predicted substrates for these enzymes are 3-OH palmitoyl-acyl carrier protein (3-OH palmitoyl-ACP), C6- and C8-acylated-acyl carrier protein (acyl-ACPs), and S-adenosylmethionine (AdoMet). AidA is a protein of unknown function; its structural gene is regulated by SolR in response to sufficient concentrations of AHLs. Only the circuits that alter traits by more than fivefold are shown. Dashed lines with hatched arrowheads represent diffusion of signal compounds into and out of the cell. Open arrowheads represent perception of extracellular signals by two-component sensors; filled arrowheads represent transfer of phosphate from sensor proteins to response regulators. Lines with filled arrowheads or bars represent positive or negative control, respectively. Unlike most two-component response regulators, which activate transcription of target promoters in response to a signal, PhcR acts as a repressor. When concentrations of 3-OH PAME are low, it is hypothesized that PhcS phosphorylates PhcR and then PhcR~P reduces the amount of functional PhcA. When the concentration of 3-OH PAME exceeds a threshold, it likely interferes with the phosphotransfer functions of PhcS, thus relieving repression by PhcR. For additional details and references see Schell (1996), Clough et al. (1997b), and Flavier et al. (1997a, 1997b).

Conclusions

That virulence genes are so highly regulated likely indicates how important it is for them to be expressed only at an appropriate time or situation. *Ralstonia solanacearum* presumably has evolved to recognize selected environmental signals because they are important for its survival or successful colonization of a host plant. Thus, by identifying the signals and dissecting the regulatory network that controls virulence genes we should learn much about survival and pathogenesis of *R. solanacearum*. Such knowledge may also lead to new strategies for controlling the pathogen.

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Biocontrol: genetic modifications for enhanced antifungal activity

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Abstract

Pseudomonas fluorescens strain BL915 is an effective biocontrol agent for the control of plant diseases caused by *Rhizoctonia* and *Pythium*. The overall biocontrol activity of this strain is due mainly to the production of secondary metabolites and proteins with antifungal activities. These products include pyrrolnitrin (Prn), chitinase, HCN, 2-hexyl-5-propyl resorcinol (Res), and gelatinase. The synthesis of these compounds is coordinately regulated by *lemA* and *gacA* homologs. We have cloned and characterized a 4 gene cluster from strain BL915 responsible for Prn biosynthesis. Mutation of any one of the genes resulted in a Prn-nonproducing phenotype and a reduction in biocontrol activity. The *tac* promoter was inserted in front of the Prn gene cluster and integrated into the chromosome resulting in constitutive Prn production. Aditionally the tac/Prn gene fusion was mobilized on a broad host range plasmid into the wt strain. In both cases Prn production and biological activity was increased. A mutant unable to produce Res was shown to produce more Prn and had increased biocontrol activity. This is most likely due to competition of precursors derived from shikimate. The introduction of additional plasmid-borne copies of the *gacA* gene homolog into strain BL915 resulted in the production of increased amounts of *gacA*-regulated metabolites and in increased biocontrol activity. Similarly, both the *lac* and *tac* promoters were integrated in front of the *gacA* gene resulting in improved biocontrol efficacy.

Biological control systems

Biological Control of Fungal and Bacterial Plant Pathogens
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Biochemical and MRFLP genomic characterization of antagonistic *Pseudomonas fluorescens* involved in biological control of brown spot of pear

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Abstract

Several strains of *Erwinia herbicola* and *Pseudomonas fluorescens* antagonistic to *Stemphylium vesicarium*, the causal agent of brown spot of pear, were isolated from a very wide range of environments. Strains were evaluated for in vitro antagonism against eleven fungal and bacterial plant pathogens and for their ability to inhibit infection of *S. vesicarium* and *Erwinia amylovora* on pear. Strains were also studied in relation to the utilization of 95 organic carbon sources with the Biolog system. Banding patterns of MRFLP of genomic DNA with *Swa*I in 34 selected *P. fluorescens*, including antagonists and non-antagonists of *S. vesicarium*, were studied with Pulsed Field Gel Electrophoresis. Twenty-eight different patterns were identified with a very high heterogeneity among strains. Cluster analysis indicated that there were no relationships between clusters obtained with biovar type, host and plant part of origin, pathogen antagonism in vitro, inhibition of infection by *S. vesicarium* in planta, Biolog profiles, or MRFLP patterns.

Introduction

The most economically important pear pathogens in commercial orchards in many areas of Europe are the bacteria *Pseudomonas syringae* and *Erwinia amylovora*, and the fungus *Stemphylium vesicarium* (López et al. 1996, Montesinos and Vilardell 1991, Montesinos et al. 1996). Control of these diseases is achieved by means of several chemical antimicrobial sprays during the growing season. However, intensive treatments may cause undesirable non-target effects in the pathogen (eg., development of resistance), the environment, and to pear consumers. Integrated pear production in Catalunya, Spain and other parts of Europe is expanding but is limited by the need of use of chemical bactericides and fungicides. It is expected that biological control of these diseases could be an alternative or complementary method to chemical control and could reduce the use of chemical pesticides in pear production.

The objective of this work was to screen a diversity of environments (i.e., plant hosts, plant parts, and geographical locations) for the presence of *E. herbicola* and *P. fluorescens* antagonistic to pear pathogens, to evaluate their potential biocontrol activity, and to study their biochemical and genomic heterogenity.

Materials and Methods

Samples from aerial plant surfaces and root systems were taken from several locations and different plant species in Northeastern Spain during the growing seasons of 1993 and 1994. Epiphytic strains of *E. herbicola* (syn. *Enterobacter agglomerans*) and *P. fluorescens* were recovered by washing plant material and plating onto King's B (KB) and Miller-Schroth agar (Montesinos et al. 1996). In vitro antagonism against the pear pathogens *E. amylovora*, *P. syringae,* and *S. vesicarium* was studied on Luria-Bertani (LB), KB, glucose-asparagine (GA), and potato-dextrose (PD) agar. The spectrum of in vitro antagonism against eleven fungal and bacterial plant pathogens was also studied. Antagonism in plant tissues was determined using infection inhibition bioassays consisting of a detached leaf test for *S. vesicarium* and an immature pear disc test for *E. amylovora* and *P. syringae*. The nutritional requirements of antagonistic strains to *S. vesicarium* in relation to 95 carbon sources were analyzed with the Biolog system and compared to *S. vesicarium* requirements using a coincidence index (Janisiewicz 1996). The genome of 34 strains of *P. fluorescens* antagonistic and non-antagonistic to *S. vesicarium* was characterized analyzing macro-restriction fragments of DNA obtained with *Swa*I low frequency target restriction enzyme and separated by Pulsed Field Gel Electrophoresis (PFGE) following a modification of methods described by Römling et al. (1989).

Culture supernatants of *P. fluorescens* EPS288 were studied for antifungal activity in relation to culture media composition, temperature, pH, and cell concentration. Supernatants were concentrated by organic solvent extraction and studied by TLC and HPLC coupled to conidial germination inhibition bioassays. Cyanide production was detected by the alcaline picrate or spot test methods (Castric and Castric 1983) and quantitatively by standard methods (APHA-AWWA-WEF 1992).

Results and Discussion

Isolates conforming to the *P. fluorescens* and *E. herbicola* species were chosen for antagonism studies. A total of 404 isolates were characterized including 212 *P. fluorescens* and 192 *E. herbicola* isolates. Most of the *P. fluorescens* isolated from roots were of biovar V, whereas, those isolated from aerial plant parts were of biovar V and biovar I. Most of the *E. herbicola* isolates belonged to biogroup 1.

Antagonism depended on the growth medium and on the pathogen. The GA medium showed the highest frequency of antagonistic strains. The antagonistic pattern on LB, KB, PD, and GA against the three pear pathogens was used for grouping the strains. The percentage of antagonists was higher among isolates from roots than from aerial plant parts. A higher frequency of isolates of *P. fluorescens* than of *E. herbicola* were antagonistic on GA. *Erwinia amylovora* was inhibited by more isolates than *P. syringae*, and *P. syringae* was inhibited by more isolates than *S. vesicarium* (Fig. 1).

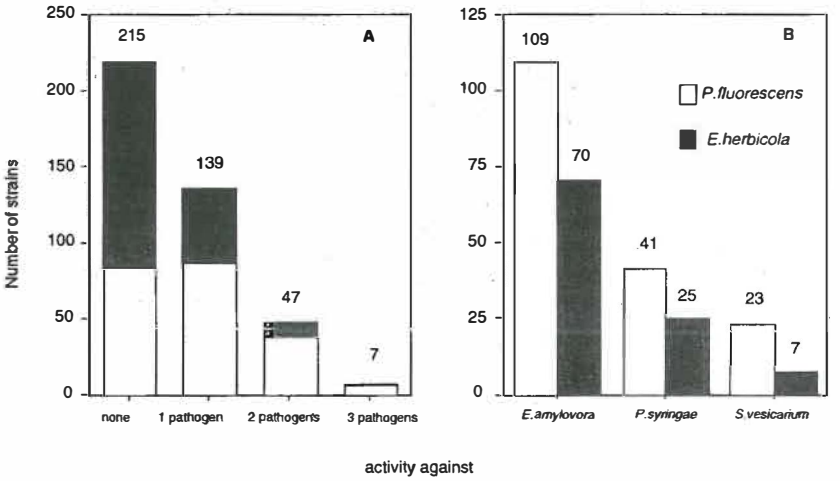

Fig. 1. Distribution of strains of *Erwinia herbicola* and *Pseudomonas fluorescens* isolated from several plant parts, host plants and geographic locations, in relation to the antagonistic potential against none, 1, 2 or 3 pathogens (A), and against *E. amylovora*, *P. syringae* and *Stemphylium vesicarium* (B).

The in vitro activity against three plant pathogenic bacteria and eight plant pathogenic fungi was studied in 28 strains of *P. fluorescens* and *E. herbicola* antagonistic to *S. vesicarium* (Fig. 2). Cluster analysis revealed seven main groups of strains at a cutting distance of 0.85 characterized by the following activities: strong antifungal and antibacterial (strain Pf372), strong antifungal (strain Pf288), antifungal (cluster 3), antibacterial and weak antifungal (cluster 4), only antibacterial (strain En 513), weak but wide spectrum (cluster 6), and weak antifungal (cluster 7). No relationship was observed between in vitro antagonism patterns and the capacity of the strains for inhibition of infection by *S. vesicarium* or *E. amylovora* in planta. The strains were subjected to a nutritional study using 95 carbon sources. *Erwinia herbicola* and *P. fluorescens* were separated at a cutting level of 68% similarity and several clusters were obtained within each species. However, no relationship was observed between Biolog profiles and biovar, host and plant part of origin, and antagonism pattern against *P. syringae, S. vesicarium,* and *E. amylovora*.

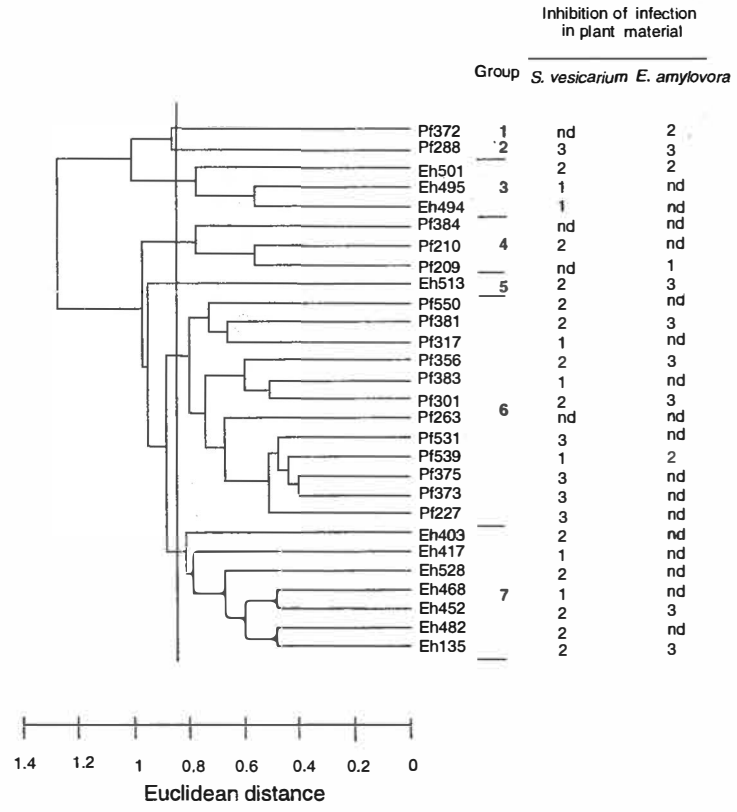

Fig. 2. Cluster analysis of the antagonism spectrum against 11 fungal and bacterial plant pathogens of strains of *Erwinia herbicola* and *Pseudomonas fluorescens* inhibiting *Stemphylium vesicarium*. Patterns are compared to the potential for inhibition of infection by *S. vesicarium* and *E. amylovora* in plant material. nd = not determined. Groups 1, 2, and 3 had low, moderate, and high inhibition of infection.

The DNA banding patterns of 34 strains of *P. fluorescens* obtained after digestion of the genome with *Swa*I and PFGE were used for cluster analysis and related to host and plant part of origin, biovar and antagonism spectrum (Fig. 3). Twenty-eight banding patterns with bands sizing between 51 and 648 Kb were observed. More than 50% of the genome was separated in 18 strains, 30 to 50% in 15 strains, and only 7% in strain EPS317. Similarity was very low (mean of 20%) indicating great heterogeneity among strains. No relationship was found between *Swa*I PFGE-MRFLP patterns and biovar, antagonism spectrum, plant host or plant part of origin. These results agreed with other reports on fluorescent pseudomonads isolated from field sites (Rainey et al. 1994).

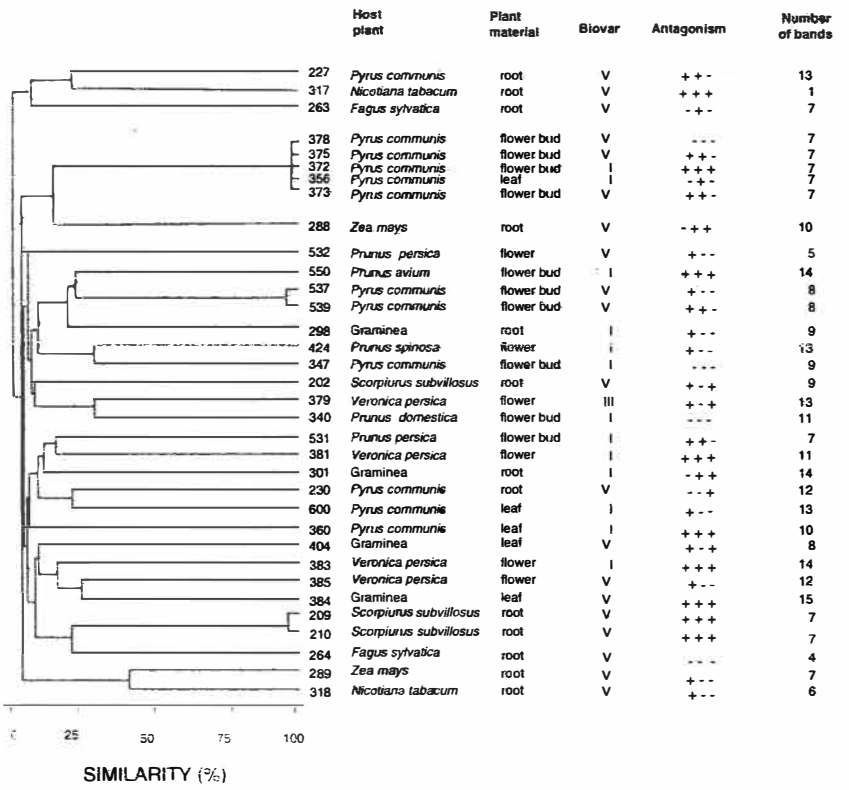

Fig. 3. Cluster analysis of the banding patterns of genomic DNA of selected *Pseudomonas fluorescens* strains, antagonistic and non-antagonistic to *Stemphylium vesicarium*, obtained by digestion with *Swa*I and separation by Pulsed Field Gel electrophoresis. Patterns are compared to host and plant material of origin, biovar, and antagonism against *P. syringae*, *S. vesicarium*, and *Erwinia amylovora*.

Among the studied strains *P. fluorescens* EPS288 was the most effective antagonist and gave biocontrol levels of brown spot on pear plants under greenhouse trials similar to the fungicide captan (Montesinos et al. 1996). The strain clearly showed singular antagonism and MRFLP patterns. Cell-free supernatants of EPS288 were highly antifungal and inhibited conidial germination of *S. vesicarium*. Optimum conditions for antifungal activity were found at 10 to 30 °C, pH 5 to 7, and in culture media without sugar but rich with amino acids. Antifungal activity of liquid culture supernatants of strain EPS288 was lost during organic solvent extraction and manipulation. Strain EPS288 produced hydrogen cyanide in liquid culture starting at a cell concentration of 10^8 CFU/ml with maximum production at the transition from exponential to stationary phase but continuing for more than five days. The total amount of hydrogen cyanide recovered by flushing with an air stream was 40 mg/l of broth. Our results with strain EPS288 were similar to those obtained with *P. fluorescens* CHA0 which suppresses black root rot of tobacco and other plant diseases (Voisard et al. 1989).

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biological control of *Cercospora beticola* on sugar beet with phyllosphere bacteria

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Abstract

Cercospora leaf spot (CLS) has become more difficult to control because of the occurrence of fungicide resistance/tolerance in the pathogen population, and the potential loss of fungicides due to regulatory action of the US Environmental Protection Agency. Two phyllosphere bacteria (J and B) which produced chitinase and ß-1,3-glucanase, and exert direct antibiosis in vitro against *C. beticola* were tested in growth chamber experiments for control of CLS. Both strains showed up to 85% disease control. To enhance survival of the bacteria in the phyllosphere they were supported with 1% ß-glucan as a selective supplemental food base. The efficient disease control with strains B and J in growth chamber experiments and J in field trials with cell densities of log 4 to log 5 per gram fresh leaf weight indicated other mechanisms may be involved than direct antibiosis or hyperparasitism. In growth chamber experiments with the systemic inducer benzothiadiazole (BION®, Novartis, Basel, Switzerland) and isolates B and J, all induced systemic resistance when applied on a separate leaf three days prior to challenge inoculation with *C. beticola*. Pre-inoculation with *C. beticola* did not reduce disease severity after challenge inoculation with the pathogen.

Introduction

Cercospora leaf spot (CLS) caused by *Cercospora beticola* is of great importance in the sugar beet production areas in the United States, resulting in loss of income for the farmers through reduction of sugar yield and quality. In the past, CLS has become more difficult to control because of the occurrence of fungicide resistance/tolerance in the pathogen population. The objectives of our studies were (i) to develop effective biological control for CLS with phyllosphere bacteria (ii) evaluate the potential of combinations of bacteria and fungicides for synergism and resistance management (iii) and to investigate the modes of action involved in biological control. The efficacy of phyllosphere bacteria (isolate J) for control of CLS in field trials has been demonstrated (Jacobsen et al. 1997). In this paper we present examples for synergism of bacteria with fungicides and data that suggest the possible involvement of systemic induced resistance as a mode of action besides direct antibiosis and hyperparasitism in the biological control of CLS.

Materials and Methods

Synergism between bacteria and fungicides was evaluated. A commercial freeze dry formulation of both bacteria (isolate J and B) was used in this experiment. Eight weeks old sugar beet plants (cultivar HH88) were sprayed with J and B suspended in 50 ml of a 0.1% methyl cellulose solution (MCS) in concentrations of log 8 and log 7 until run-off with 5 plants per treatment. The fungicides TILT® (propiconazole, Novartis) and RH7592

(fenbuconazole, Rohm and Hars) were applied the same way using MCS as formulation in concentrations of 150 ppm and 90 ppm (product), respectively. Additionally, J and B were sprayed with RH7592 (90 ppm). Immediately after application, plants were transferred to a growth chamber (28 °C, 16 h photoperiod) and inoculated with a mycelial suspension of *C. beticola*. Approximately 250 ml of this suspension (log 5 CFU/ml) was used to inoculate 100 plants. The plants were incubated for 4 days in a mist chamber with humidifiers and a leaf wetness period of at least 14 h per day.

Isolates J and B were grown in 50 ml Tryptic Soy Broth (TSB) on a rotary shaker. After 24 h at 28 °C, the broth was centrifuged and cells washed in sterile phosphate buffer. After a second centrifugation cells were resuspended in 50 ml sterile MCS. The resulting suspension contained approximately log 8 CFU/ml. The systemic inducer BION® (benzothiadiazole) was also suspended in 50 ml of MCS at a concentration of 200 ppm (product). One leaf per single plant was sprayed until run-off with bacteria, BION®, or a suspension of *C. beticola* containing log 5 CFU/ml, respectively and covered immediately with plastic bags to avoid spread of bacteria on other leaves. Plants were kept in a growth chamber at 28 °C for 3 days followed by challenge inoculation of the untreated leaves with a *C. beticola* suspension and incubated in a mist chamber as described above. A total of 8 (exp. 2) and 10 (exp. 3) plants were used for each treatment. For all experiments, disease development was rated according to the KWS-scale (0 = no lesions visible and 10 = 50% disease severity) between 14 and 28 days past inoculation (DPI) with the pathogen.

Results

At the first disease evaluation, 17 DPI, all treatments significantly reduced severity of CLS (Table 1). Application of isolates J and B in commercial formulation resulted in up to 83.1% reduction of CLS. TILT® reached only 56%. Combinations of bacteria with RH7592 gave the best control with 99.4 and 96.8% reduction, respectively, compared to fungicide alone with 91.4%. A second disease rating at 28 DPI showed that TILT® was no longer effective in controlling CLS. Isolates J and B still significantly reduced CLS between 52 and 67%. However, for both bacteria no significant differences between log 8 and log 7 application rates were observed. Combination of isolate J with RH7592 gave the best control of CLS with 91.8% reduction compared to fungicide alone with 73.2%.

Treatment with either BION® or isolates J and B resulted in significantly lower disease severity compared to the untreated control 14, 21, and 28 DPI, respectively (Table 2). Pre-treatment with *Cercospora* showed in both experiments no effect on disease development. However, treatment with isolate B resulted in a significantly lower disease severity at 28 DPI and AUDPC compared to the systemic inducer BION®. In the second experiment with only two disease readings at 14 and 28 DPI all treatments showed again significant disease reduction compared to both controls (Table 2). Treatment with bacteria resulted in better disease control when compared to the systemic inducer BION®, with up to 79.5% reduction. Furthermore, treatment with BION® resulted in phytotoxicity in sugar beet plants, visible 14 days after application.

Table 1. Effect of phyllosphere bacteria (isolate J and B) with two rates of commercial formulation, TILT®, RH7592, and combinations of bacteria with RH7592 on disease severity of Cercospora leaf spot on sugar beet 14 and 21 DPI.

Treatment (CFU/ml)	14 DPI		21 DPI	
	% disease severity	% reduction	% disease severity	% reduction
Control-untreated	16.5 a	-	28.6 a	-
TILT®	7.2 b	56.1	24.6 a	14.2
RH7592	1.4 de	91.4	7.7 bcd	73.2
Isolate J (log 8)	5.2 bc	68.2	9.3 bc	67.4
Isolate J (log 7)	3.2 cd	80.3	13.6 b	52.5
Isolate J (log 8) plus RH7592	0.1 e	99.4	2.3 d	91.8
Isolate B (log 8)	2.8 cd	83.1	11.4 bc	60.2
Isolate B (log 7)	3.2 cd	80.5	9.9 bc	65.4
Isolate B (log 8) plus RH7592	0.5 de	96.8	6.2 cd	78.4

Means in one column followed by the same letter are not significantly different at $P = 0.05$ (n = 5); DPI = days past inoculation with a C. beticola suspension containing log 5 CFU/ml

Table 2: Effect of BION® and phyllosphere bacteria (isolate J and B) on disease severity and AUDPC of Cercospora leaf spot on sugar beet 14, 21 and 28 days past challenge inoculation with CLS in two growth chamber experiments.

Experiment 1:

Treatment	% disease severity			
	14 DPI	21 DPI	28 DPI	AUDPC
Control-untreated	9.95 a	29.12 a	32.58 a	352.7 a
Control-*Cercospora*	9.10 ab	31.96 a	36.36 a	382.8 a
BION®	3.97 cd	14.42 b	19.39 b	182.7 b
Isolate J	5.84 bc	13.75 b	15.33 bc	170.4 bc
Isolate B	1.83 d	11.19 b	12.90 c	129.9 c
LSD 5%	3.65	5.04	4.89	46.5

Experiment 2:

Treatment	% disease severity		
	14 DPI	21 DPI	% reduction
Control-untreated	5.76 a	14.34 a	-
Control-*Cercospora*	5.24 a	14.10 a	-
BION®	0.76 b	5.26 b	63.6
Isolate J	1.03 b	2.94 b	79.0
Isolate B	0.42 b	3.43 b	76.0
LSD 5%	2.87	3.78	n.d.

DPI = days past challenge inoculation with a *C. beticola* suspension containing log 5 cfu/ml; AUDPC = Area under the disease progress curve based on the three evaluations 14, 21 and 28 DPI; Cercospora, BION®, J and B were applied to a single leaf 3 days prior to challenge inoculation with CLS; Means in one column followed by the same letter are not significantly different at $P = 0.05$ (n = 8; n = 10)

Discussion

The current and previous studies (Jacobsen et al. 1997) demonstrate the potential of phyllosphere bacteria for control of *Cercospora* leaf spot on sugar beet. Two phyllosphere bacteria (isolate J and B) produced chitinase and ß-1,3-glucanase, and inhibited *C. beticola*. The synergism observed when fungicides were applied in combination with bacteria in growth chamber experiments was confirmed in field experiments in 1996 (Jacobsen et al. 1997). To enhance survival of the bacteria in the phyllosphere under field conditions they were supported with a 1% ß-glucan solution as a supplemental food base.

The results of the growth chamber experiments suggest that systemic induced resistance may contribute to the biological control of CLS. Nielsen et al. (1993) demonstrated the importance of increased chitinase activity as a defense system of sugar beet against CLS. Experiments are currently underway to investigate the possibilities of a plant defense system triggered by the phyllosphere bacteria. Population dynamic studies in the growth chamber with rifampicin resistant mutants of both strains showed population densities between log 5 and log 4 per gram fresh leaf weight two days past inoculation with *C. beticola* (Kiewnick et al. *unpublished data*). This also indicated other mechanisms involved in control of CLS than direct growth inhibition and hyperparasitism.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biological control of *Fusarium* spp. by a maize rhizosphere population of *Burkholderia cepacia*

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Abstract

In order to select bacteria with potential for controlling maize diseases, a *Burkholderia cepacia* population isolated from maize rhizosphere was characterized for the ability to antagonize and repress, under in vitro and greenhouse conditions, *Fusarium proliferatum* ITEM-381 and *F. moniliforme* ITEM-504, both isolated from maize tissues in Italy. The in vitro test showed that all the *B. cepacia* isolates were able to restrict the growth of the two *Fusarium* strains, exerting a generally higher antagonistic effect against *F. proliferatum* ITEM-381 than *F. moniliforme* ITEM-504. In addition, the fungal inhibition was higher on KB rather than PDA plates, suggesting that siderophore production could be involved in the antagonistic response. Greenhouse experiments, performed in soil artificially infested with *F. proliferatum* ITEM-381 or *F. moniliforme* ITEM-504, indicated that 40% and 53% of *B. cepacia* isolates tested have a potential for controlling maize diseases caused by *F. proliferatum* ITEM-381 and *F. moniliforme* ITEM-504, respectively.

Introduction

Fungal diseases of maize (*Zea mays* L.) occur world-wide and cause significant yield losses. Both seedlings and mature plants are attacked by a range of soil-borne or seed-borne fungi which invade through natural openings or wounds, causing seed rots, seedling blight and root rots. *Fusarium proliferatum* (T. Matsushima) Nirenberg and *F. moniliforme* (J. Sheldon) are the most common fungi associated with corn. They exist as saprophytes in moribund tissues of living hosts (Hornby and Ullstrup 1967) or as opportunistic pathogens awaiting stress in the host (Wall and Mortimore 1965). *Fusarium proliferatum* can be isolated from maize tissues including roots, stalks, tassels, silks and kernels, can survive in maize field soil, probably as thickened hyphae, and is transmitted through maize seeds, as is *F. moniliforme,* although at a lower percentage. *Fusarium moniliforme* is associated with root, stalk, and ear rots; moreover, this species can be isolated from all plant tissues. These two *Fusarium* species found in maize are involved in animal mycotoxicoses and are known to produce several mycotoxins, including moniliformins, fumonisins, and beauvericin (Bottalico et al. 1989). In addition, some strains of *F. moniliforme* are the industrial source of gibberellic acid (Jefferys 1970).

Fungal diseases of maize are usually controlled by a combination of cultural practices, use of fungicides, and host plant resistance. However, growing ecological problems, including health risks associated with human exposure, ground water contamination, and selection of pathogens with chemical resistance, are associated with the use of agrochemicals. Recently, there has been an increasing interest in using beneficial microorganisms as biocontrol agents of plant diseases (Weller 1988). Bacteria belonging to the species *Burkholderia cepacia* are

receiving increased attention due to their ability to antagonize and repress all the major soil-borne fungal pathogens of maize belonging to the genus *Fusarium* (Hebbar et al. 1992a), and to promote plant growth (Hebbar et al. 1994, Tabacchioni et al. 1993). In addition, it has been observed that *B. cepacia* is associated in large numbers with maize roots, representing over 4% of the total culturable bacteria present in the rhizosphere of maize. Moreover, when used as a seed inoculant, *B. cepacia* colonizes maize roots better than other bacterial antagonists (Hebbar et al. 1992b).

Root-associated bacteria are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by pathogens. Therefore, it becomes of particular importance to characterize the microorganisms naturally associated with the root system of maize, in order to select bacteria with potential for controlling maize diseases caused by phytopathogenic fungi. The objective of this study was to characterize a *B. cepacia* population isolated from maize rhizosphere in Italy, with regard to its ability to antagonize and repress, under in vitro and greenhouse conditions, two *Fusarium* species isolated from maize in Italy, *F. proliferatum* ITEM-381 and *F. moniliforme* ITEM-504.

Materials and Methods

Fusarium proliferatum ITEM-381 and *F. moniliforme* ITEM-504, isolated from preharvest maize ear and stalk rots, respectively, were provided from the collection of the Istituto Tossine e Micotossine da Parassiti Vegetali, CNR Bari, Italy. *Fusarium proliferatum* ITEM-381, mating population D, produces fumonisin B_1, beauvericin, and fusaproliferin, *F. moniliforme* ITEM-504, mating population A, produces fumonisin B_1 and fumonisin B_2 Mycelia and conidia from strains grown on carnation leaf agar (Nelson et al. 1983) were freeze-stored in sterile 18% glycerol (-75 °C).

Fungal inoculum was prepared by placing 20 colonized PDA agar plugs (7-mm diameter) of each strain to an autoclaved sand-maize meal mixture (270 g of riverbed sand, 30 g of maize meal, 60 ml of distilled water) in 1-liter Erlenmeyer flasks. Each flask was incubated at 25 °C for 2 weeks, and shaken every two days to promote further colonization, as described by Nene et al. (1981). A fungus soil-mixture was prepared by hand-mixing the contents of each flask with 5 kg of nonautoclavated sand-soil mixture (1 : 5) and placed into 30 x 24 x 10 cm plastic boxes. Soil [39% clay, 14% silt, 47% sand; organic C 2.38% (wt/wt); pH 6.04; moisture holding capacity 0.28 ml/g] was collected from the upper 30 cm of the same field from which *B. cepacia* strains were isolated (see below) and mixed thoroughly with sand. After addition of fungi, the sand-soil mixture was watered and allowed to equilibrate for 1 week before use.

Burkholderia cepacia MCI strains were isolated from the rhizosphere of *Zea mays* cv. Pactol in an experimental field with no previous cropping history of maize, at Santa Maria di Galeria, Rome, Italy. After 40 days of plant growth, two plants were randomly harvested, roots were excised, and washed five times in phosphate buffered saline (PBS, Flow-Laboratories). Roots were then blended and 0.1 ml aliquots of appropriate dilutions in sterile saline solution (9 g/l NaCl) were plated onto PCAT medium (Burbage et al. 1982). *Burkholderia cepacia* colonies were white or pale yellow, smooth, with flat edges and an elevated center. After 72 h of bacterial growth at 27 °C, 15 colonies were taken up from PCAT plates and subjected to isolation on the same medium. Bacterial isolates were stored at -75 °C in 30% (vol/vol) glycerol. Bacterial identification was confirmed with the API 20NE test (Bio-Mérieux, la Balme les Grottes, Montalieu Vercieu, France) and restriction analysis

of amplified 16S rDNA (Di Cello and Fani 1996). In vitro antagonistic activity of *B. cepacia* strains was tested on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) and King's B (KB) (King et al. 1954) media as decribed by Bevivino et al. (1994).

Cultures of *B. cepacia* were grown in 100 ml of nutrient broth (NB) for about 24 h at 28 °C and 150 rpm, washed in sterile saline solution, and resuspended in 1% methyl cellulose to half of the original culture volume (50 ml; 10^{10} CFU/ml). 50 maize seeds (cv. Pactol) were submerged in 50 ml of bacterial inoculum in 200-ml Erlenmeyer flasks. Control seeds were submerged in 50 ml of 1% methyl cellulose. Flasks were incubated at 25 °C on a rotary shaker at 70 rpm for 2 h, to allow bacterial cells to adhere to seeds (approximately 10^8 CFU/seed). After incubation, excess inoculum was removed and seeds were immediately planted in the potting medium.

Greenhouse experiments. Treatments were as follows: (i) methylcellulose-treated seeds planted in non-infested soil; (ii) methylcellulose-treated seeds planted in soil artificially infested with *F. proliferatum* ITEM-381; (iii) seeds treated separately with each of 15 *B. cepacia* isolates, and planted in soil artificially infested with *F. proliferatum* ITEM-381; (iv) methylcellulose-treated seeds planted in soil artificially infested with *F. moniliforme* ITEM-504; (v) seeds treated separately with each of 15 *B. cepacia* isolates, and planted in soil artificially infested with *F. moniliforme* ITEM-504. Maize seeds were planted in the potting medium at a depth of approximately 2.5 cm and water was added to each box on alternate days. All experiments were carried out in a greenhouse at 29 °C during the day and 18 °C at night, with a 16/8 h light/dark period. After 21 days of plant growth, 25 plants for each treatment were collected, and fresh weight (f.w.) of root and shoot was calculated. Data were analyzed using one-way ANOVA (StatView 512+, BrainPower Inc., CA, USA).

Results

In vitro antagonism. The antagonistic activity of *B. cepacia* isolates against *F. proliferatum* ITEM-381 and *F. moniliforme* ITEM-504 was tested in a plate assay using KB and PDA media. The results shown in Table 1 indicate that all *B. cepacia* strains were able to restrict the growth of the two *Fusarium* isolates on both KB and PDA, although to differing degrees. The inhibition was more evident on KB, whereas on PDA the effect was generally reduced. The bacterial strains exerted a higher antagonistic effect against *F. proliferatum* than *F. moniliforme*. On KB, 11 *B. cepacia* strains showed a percentage of inhibition of *F. proliferatum* above 70%, whereas the percentage of inhibition of the 15 isolates tested against *F. moniliforme* ranged from 54% to 62%. On PDA, all the *B. cepacia* strains inhibited *F. proliferatum* with an antagonistic activity above 50% (ranging from 50% to 64%), whereas only 6 strains showed a level of antagonism against *F. moniliforme* above 50% (ranging from 50% to 55%).

Effect of fungal inoculum on plant growth. Five experiments were conducted under greenhouse conditions to test the effect of the two *Fusarium* isolates on root and shoot fresh weight of maize, after 21 days of plant growth. Variances between experimental trials were homogeneous, and, thus, data from repeated experimental trials were pooled and analyzed using one-way ANOVA. The introduction of *F. proliferatum* ITEM-381 into the potting medium caused a significant ($P < 0.001$) reduction in both root and shoot fresh weight, as compared to control plants growing in non-infested soil (Table 2). The inoculation with *F. moniliforme* ITEM-504 caused a significant ($P < 0.05$) reduction in root fresh weight only (Table 2).

Table 1. Antagonistic activity of *B. cepacia* strains against *F. proliferatum* ITEM-381 and *F. moniliforme* ITEM-504 on KB and PDA media, measured as percentage inhibition of the fungal growth.

	F. proliferatum		*F. moniliforme*	
Strains	KB	PDA	KB	PDA
MCI 2	70.27	55.25	55.88	50.01
MCI 4	73.68	59.45	59.46	47.06
MCI 7	72.97	52.63	58.97	48.04
MCI 11	77.14	54.05	60.53	49.52
MCI 12	74.28	60.52	58.33	48.65
MCI 13	64.86	56.75	60.01	49.22
MCI 14	66.67	56.42	60.53	50.15
MCI 15	76.32	57.89	62.50	49.65
MCI 16	70.32	52.77	58.78	52.63
MCI 18	70.86	50.01	58.92	52.78
MCI 19	70.01	60.52	59.98	51.35
MCI 22	68.02	54.02	57.82	47.12
MCI 23	71.05	64.10	60.71	55.01
MCI 26	67.57	55.55	55.56	38.46
MCI 27	71.15	52.13	54.05	47.37

Table 2. Effect of *F. proliferatum* ITEM-381 and *F. moniliforme* ITEM-504 on root and shoot fresh weights of maize in greenhouse experiments.

Treatments	Roots (g)[Y]	Shoots (g)[Y]
control[Z]	1.31 ± 0.60	1.96 ± 1.01
F. proliferatum	0.97 ± 0.71**	1.49 ± 0.98**
control[Z]	1.17 ± 0.62	1.53 ± 1.29
F. moniliforme	1.00 ± 0.67*	1.52 ± 1.28

[Y] Values are the mean ± standard deviation (SD) of five experiments, each with 25 replicates.Variances between experimental trials were homogeneous, thus, data from repeated experimental trials were pooled and analyzed using one-way ANOVA; * indicates $P < 0.05$; ** indicates $P < 0.001$, according to Fisher's protected LSD.

[Z] control = maize seeds planted in non infested soil.

Biological control of *Fusarium* spp. by *B. cepacia* isolates. Greenhouse experiments were performed to test the efficacy of the bacterial isolates as biocontrol agents of *F. proliferatum* ITEM-381 and *F. moniliforme* ITEM-504. In soil infested with *F. proliferatum* ITEM-381, treatment of maize seeds with *B. cepacia* MCI 12, 15, and 27 resulted in a significant increase in both root and shoot fresh weight, seed bacterization with MCI 2, and 16 significantly increased the root fresh weight only, and seed treatment with MCI 26 resulted in significantly higher values of shoot fresh weight only (Table 3). In soil infested with *F. moniliforme* ITEM-504, *B. cepacia* isolates MCI 23, 26, and 27 exerted a positive effect on both root and shoot fresh weight, *B. cepacia* isolates MCI 14, 18, and 19 on root fresh weight only, whereas *B. cepacia* isolates MCI 13, and 16 exerted a positive effect on shoot fresh weight, only (Table 4). Seed bacterization with *B. cepacia* MCI 12 resulted in significantly lower fresh weight value of plant roots, as compared to the untreated control.

Table 3. Effect of *B. cepacia* strains on root and shoot fresh weight of maize grown in soil artificially infested with *F. proliferatum* ITEM-381.

Treatments	Roots (g)[Y]	Shoots (g)[Y]
control[Z]	0.76 ± 0.37	1.02 ± 0.62
MCI 2	1.15 ± 0.36*	1.14 ± 0.77
MCI 4	0.98 ± 0.61	1.44 ± 0.86
MCI 7	0.71 ± 0.48	1.34 ± 1.04
MCI 11	0.93 ± 0.57	1.04 ± 0.64
MCI 12	1.23 ± 0.30**	2.29 ± 0.79***
MCI 13	0.81 ± 0.54	1.60 ± 0.73
MCI 14	0.73 ± 0.53	1.04 ± 0.83
MCI 15	1.14 ± 0.31*	2.35 ± 0.92***
MCI 16	1.13 ± 0.32*	1.25 ± 0.98
MCI 18	0.90 ± 0.57	1.00 ± 0.78
MCI 19	0.42 ± 0.20	1.23 ± 1.10
MCI 22	0.97 ± 0.62	1.42 ± 0.83
MCI 23	0.38 ± 0.29	0.62 ± 0.37
MCI 26	0.85 ± 1.05	1.70 ± 0.88*
MCI 27	1.38 ± 0.39***	2.88 ± 0.91***

[Y] Values are the mean ± standard deviation (SD) of two experiments, each with 25 replicates; * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$, according to Fisher's protected LSD.
[Z] control = 1% methyl cellulose-treated seeds.

Table 4. Effect of *B. cepacia* strains on root and shoot fresh weight of maize grown in soil artificially infested with *F. moniliforme* ITEM-504.

Treatments	Roots (g)[Y]	Shoots (g)[Y]
control[Z]	0.95 ± 0.51	1.26 ± 0.66
MCI 2	0.79 ± 0.33	0.96 ± 0.37
MCI 4	0.99 ± 0.41	1.50 ± 0.62
MCI 7	0.99 ± 0.56	0.96 ± 0.38
MCI 11	0.77 ± 0.26	1.29 ± 0.57
MCI 12	0.70 ± 0.26*	1.21 ± 0.60
MCI 13	1.10 ± 0.36	1.69 ± 0.67*
MCI 14	1.22 ± 0.39*	1.41 ± 0.68
MCI 15	0.73 ± 0.37	1.26 ± 0.55
MCI 16	1.07 ± 0.41	1.77 ± 0.58**
MCI 18	1.38 ± 0.42***	1.38 ± 0.51
MCI 19	1.26 ± 0.61*	1.35 ± 0.60
MCI 22	0.76 ± 0.28	1.15 ± 0.52
MCI 23	1.27 ± 0.40*	1.95 ± 0.68***
MCI 26	1.26 ± 0.39*	1.67 ± 0.65**
MCI 27	1.21 ± 0.41*	1.63 ± 0.69*

[Y] Values are the mean ± standard deviation (SD) of two experiments, each with 25 replicates; * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$, according to Fisher's protected LSD.
[Z] control = 1% methyl cellulose-treated seeds.

Discussion

Selection of effective bacterial strains prior to testing under commercial conditions is the major bottleneck in the development of useful biocontrol agents. *B. cepacia* has been shown to protect against or decrease the severity of various crop diseases (McLoughlin et al. 1992); in particular, when used as seed inoculant, this species has been reported to reduce maize seedling infection of *F. moniliforme* (Hebbar et al. 1992a, Hebbar et al. 1994). Since this species competes, survives and colonizes roots of various maize cultivars in a wide range of soil types, and has been shown to promote plant growth, we sought to characterize a population of *B. cepacia* naturally present in maize rhizosphere. The present study indicates that maize-rhizosphere isolates of *B. cepacia* are promising biocontrol agents of pathogenic strains of *Fusarium*.

All 15 *B. cepacia* isolates were able to restrict the in vitro growth of both *Fusarium* strains, showing differences in the level of antagonism. The antagonistic activity was affected by the medium composition, as it was higher on KB (an iron-limiting medium which stimulates production of siderophores) rather than PDA (a medium with sufficient endogenous iron to prevent siderophore production) plates. So, it can be hypothesized that siderophores, in addition to antibiotics, could be involved in biological control exerted by *B. cepacia* strains. In addition, all the bacterial isolates showed a higher antagonistic effect against *F. proliferatum* ITEM-381 than *F. moniliforme* ITEM-504.

It is generally recognized that expression of antagonism by a microorganism towards a pathogen in culture media cannot be considered evidence that the microorganism will have a functional role in controlling the pathogen in the field (Reddy and Hynes 1994). This is not unexpected, as the nutrional environment and many other factors that affect growth and survival of biocontrol agents in nature are considerably different from those in nutrient-rich culture media. Therefore, a pot screening designed to test the biological control activity of a *B. cepacia* population against two *Fusarium* strains was carried out. Our study indicated that antagonism recorded in vitro did not entirely correlate with the effectiveness of *B. cepacia* isolates to reduce the infection due to *Fusarium* spp. in the greenhouse. On the contrary, the biotest carried out in vivo with young maize plants allowed to select only one strain with a positive effect on both root and shoot fresh weight of maize in soil infested with both *F. proliferatum* and *F. moniliforme*, two strains with a positive effect on root and/or shoot fresh weight of maize in soil infested with both *F. proliferatum* and *F. moniliforme*, six MCI strains with a positive effect on root and/or shoot fresh weight of maize in soil infested with *F. proliferatum*, and eight MCI strains with a positive effect on root and/or shoot fresh weight of maize in soil infested with *F. moniliforme*.

In conclusion, 40% and 53% of *B. cepacia* isolates tested have a potential as maize inoculants for the biological control of *F. proliferatum* ITEM-381 and *F. moniliforme* ITEM-504, respectively. However, further research is needed to get a better insight into the interactions between *Fusarium* spp. and the *B. cepacia* strains with a generally higher biological control activity. We will investigate the influence of the presence of various *Fusarium* species on population dynamics and genetic diversity of *B. cepacia*. A more complete comprehension of dynamics of bacterial rhizosphere populations is an important factor for a more effective management of biocontrol microbial agents.

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Evaluation of antagonistic activity of epiphytic yeasts against rot pathogens of mandarin orange and grapefruit

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Abstract

Biocontrol activity of wide range of epiphytic yeasts against postharvest decay of citrus was evaluated on grapefruit. Antagonistic yeasts were isolated from surface of mandarin orange in Turkey and grapefruit in Israel. Their antagonistic activity against green and blue mold decay, caused by *Penicillium digitatum* and *P. italicum*, respectively, was evaluated directly on the fruit. Among the mandarin orange isolates, three were effective in inhibiting the infection of green mold. Among the grapefruit isolates, several yeasts showed high efficacy against green and blue mold and sour rot pathogens. At the wound site, the population of yeasts increased gradually and maintained high a concentration during one week of storage at 20 °C. RAPD-PCR and ap-PCR analysis indicated the presence of three groups of yeasts only.

Introduction

Postharvest decay of citrus fruit lowers fruit quality and greatly reduces yield. Green and blue molds caused by *Penicillium digitatum* (Pers) Sacc., *P. italicum* Wehmer and sour rot caused by *Geotrichum candidum* Link are the most common postharvest pathogens of citrus (Bancroft et al. 1984). Synthetic fungicides are used intensively in packinghouses to protect fruit against fungal pathogens (Eckert 1990). There is interest in alternative control methods because fungicide residues on fruit may cause public health and environmental problems and resistance may develop in pathogen populations (Wilson et al. 1991). Biological control, particularly using the yeast *Candida oleophila* in a commercially available formulation called Aspire™, has been reported to provide some protection against citrus fruit rots (Chalutz and Wilson 1990, Droby et al. 1991, Wisniewski and Wilson 1992). The objective of this study was to isolate and characterize epiphytic yeasts from citrus fruit surfaces and to screen for antagonistic activity against the fruit rot pathogens *P. digitatum*, *P. italicum*, and *G. candidum*.

Materials and Methods

Epiphytic yeast with potential antagonistic activity were isolated from surfaces of mandarin orange in Turkey in 1996 and surfaces of grapefruit in Israel in 1997. Isolates were obtained by washing fruits individually in a flask containing 200 ml sterile distilled water. The flask was placed on a rotary shaker at 100 rpm for 1 h. One hundred microliters of the washing water was then plated on nutrient yeast dextrose (NYD) agar. After 48 h of incubation at 25 °C, yeast colonies were randomly selected and streaked on NYD plates to obtain pure cultures.

Biocontrol activity of epiphytic yeasts were initially tested on grapefruits against *P. digitatum*. Yeast isolates were grown in erlenmeyer containing 25 ml NYD broth for 48 h on rotary shaker. Cultures were centrifuged at 10,000 rpm for 10 min at 4 °C and cell pellets were resuspended in equal volume of distilled water to the initial concentration. In all experiments, a concentration of 10^8 cells/ml of the yeasts was used. Fruit were surface-sterilized with 70% alcohol and wounded around the stem end at three sites. Six fruit were used for each treatment. Thirty microliters of yeast cell suspension were applied into each wound, and allowed to dry for 1 to 2 h at room temperature, and then inoculated with 20 µl of a pathogen spore suspension at concentration of 5 x 10^4 spores/ml. Sterile water was used as a control. Fruits were then covered with a high density polyethylene sleeve to maintain high moisture conditions and kept at 20 to 22 °C for 7 days. The percent of infection was calculated by counting the number of decayed wounds after 7 or 10 days of incubation. Antagonists were tested at concentrations of 10^6 to 10^8 cells/ml. *Penicillium digitatum* was inoculated at a concentration of 5 x 10^4 spores/ml.

To determine the ability of the most effective yeast antagonist to colonize the wound site of the fruit, wounds were treated with 10 µl of yeast cell suspension at concentration of 10^6 cells/ml. At various times after application of yeast cells, wounds were cut with a sharp scalple and homogenized using altrotorax in 10 ml sterile water. Three wounds obtained from each fruit were combined and used as one replicate. Four fruits (total 12 wounds) were used for each time. After serial dilutions, homoginate was plated on potato dextrose agar (PDA) containing chloramphenicol at 250 µl/ml. Populations of antagonists were determined at 0, 1, 2, 3, and 7 days. The number of colonies was counted after 48 h incubation at 20 °C.

The yeast isolates showing the highest biocontrol against *P. digitatum* were selected for further studies to evaluate their efficacy against *P. italicum* and *G. candidum* on grapefruit and *Botrytis cinerea* on grape with the same methods. *Geotrichum candidum* was applied at concentration of 5 x 10^7 spores/ml. Single grape berries were surface disinfected by dipping in 2% sodium hyphypochlorlorate, washed with tap water and allowed to dry. Berries were wounded at one site on their side by piercing with a needle. Twenty microliters of yeast cell suspension was placed into each wound, allowed to dry and inoculated with 20 µl of spore suspension of *B. cinerea* at a concentration of 5 x 10^4 spores/ml.

The characterization of yeast isolates was done by using RAPD-PCR and ap-PCR (Freeman and Shabi 1996). Genomic DNA of yeasts was extracted by using the method described by Hofmann and Winston (1987) with some modifications. One loop of yeast was taken from a colony growing on PDA plate was suspended in eppendorf tubes containing 100 µl of breaking buffer and 100 µl of phenol : chloroform : isoamin alcohol (25 : 24 : 1) to give a final concentration of 10^7 to 10^8 cells/ml and a volume of equivalent 100 µl of acid-washed glass beads were added to the solution. Eppendorf tubes were vortexed at high speed for 5 min, and then spun in a microfuge for 5 min at room temperature (Hofmann and Winston 1987). For RAPD-PCR reactions, primers of GAT GAC CGC C (OPC-05), GTC CCG ACG A (OPC-07) and ACC CGG TCA C (OPD-20) were used. For arbitrarily primed PCR (ap-PCR) primers derived from mini-satellite or repeat sequences, GACAGGACAGGACAG $(GACAG)_3$ and GACAGACAGACAGACA $(GACA)_4$ were used. PCR reactions were performed in a total volume of 20 µl, containing 0.5 µl genomic DNA, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each of the dATP, dCTP, dGTP, dTTP, 1.5 mM $MgCl_2$, 1 unit *Taq* polymerase (Promega, Madison, WI, USA) and 1 µM primer. The reactions were incubated in a Pogrammable Thermal Controller (PTC-100™, Peltier-Effect Cycling, MJ Research, USA), starting with 5 min of denaturation at 95 °C. For ap-PCR, this was followed by 30 cycles

consisting of 30 s at 95 °C, 30 s at 48 °C and 1.5 min at 72 °C (Freeman and Shabi 1996). The amplification products were separated in agarose gels (1.5%) in TAE buffer and electrophoresed at 70 V for 1.5 h. For each treatment, six fruits with three wounds each were used. Each fruit served as a replicate. Data were subjected to variance analysis and mean values were compared using Duncan's multiple range test.

Results and Discussion

Three of the yeasts (Y2, P4, P5) isolated from surfaces of mandarin orange in Turkey inhibited infection by *P. digitatum* by over 77% (Table 1). Ten yeasts isolated from surfaces of grapefruit were antagonistic to *P. digitatum*. All of the grapefruit isolates reduced green mold to 50% or less compared to 90% decay for the water control when applied at a concentration of 10^7 CFU/ml (Fig. 1). Six of the yeast completely controlled green mold when applied at a rate of 10^8 CFU/ml. For all grapefruit isolates, control was better at higher application rates. Six of the grapefruit isolates also reduced blue mold on grapefruit to 16.7% decay or less compared with 100% decay for the water control (Table 2). These six were also effective against *B. cinerea* on grape reducing decay to 23.5% or less compared with 88.9% for the water control (Table 2). Isolate H30 was very effective against sour rot caused by *G. candidum* on grapefruit (Table 2). This suggests that antagonistic yeasts have pathogen-specificity.

Table 1. Effect of yeasts isolated from the surface of mandarin orange against *Penicillium digitatum* 7 days after inoculation.

Isolate	Percent decayed wound[z]
B1	55.6 abc
Y2	22.2 bc
K3	100.0 a
P4	16.7 c
P5	22.2 c
Water control	94.4 ab

[z] Means followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

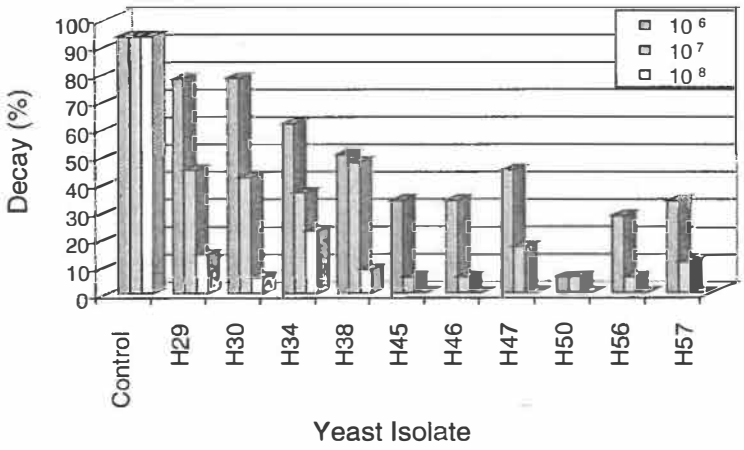

Fig. 1. Effect of cell concentration on biocontrol activity of yeast antagonists.

Table 2. Biocontrol activity of yeast against pathogenic fungi on grape[z].

Isolate	*Penicillium italicum*	*Geotrichum candidum*
H30	5.6 b	0 b
H50	5.6 b	27.8 ab
H29	11.1 b	nd
H34	16.7 b	nd
H45	5.6 b	nd
H56	11.1 b	nd
Water Control	100.0 a	50.0 a

[z] Values represent percent decayed wounds. Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.01). nd = not determined.

Two of the most effective yeasts, H30 and H50, increased and maintained high populations in surface wounds on grapefruit (Fig. 2). Both were inoculated at from 10^5 CFU/wound. Populations of H50 remained stable from 2 to 7 days at approximately 10^6 CFU/wound while populations of H30 increased steadily for 3 days to 10^6 CFU/wound and then tapered off approaching 10^5 CFU/wound at 7 days.

PCR analysis of genomic DNA indicated that white yeast isolates were distinct from pink isolates. All white yeast isolates had a PCR fingerprint similar to *Candida oleophila* isolate 182. Isolate H6 was morphologically distinct and was in a third group (Fig. 3).

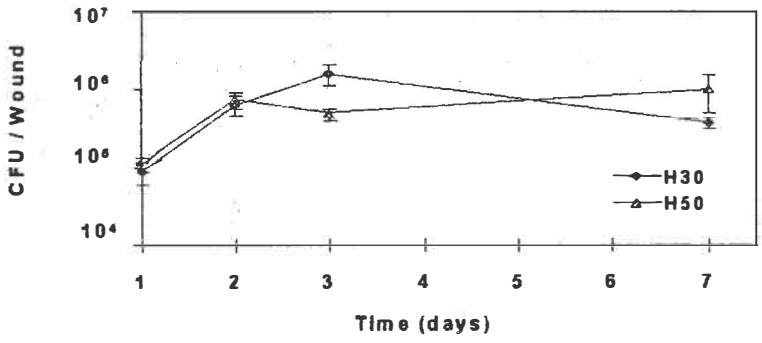

Fig. 2. Colonization of surface wounds of grapefruit by antagonistic yeasts at 20 °C. Bars indicate standard deviation of population

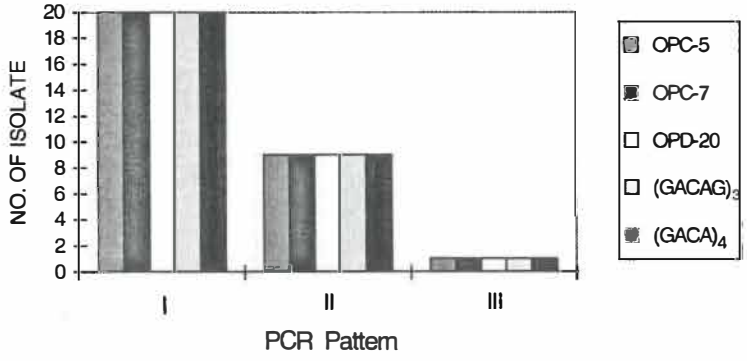

Fig. 3. Group of yeast isolates according to the banding patterns of RAPD and ap-PCR amplified genomic DNA. OPC primers were used for RAPD and other primers for ap-PCR.

Results of this study showed that a great number of yeast antagonists could be selected from the surface of the fruit. Some yeasts had high antagonistic activity against three citrus pathogens, *P. digitatum, P. italicum*, and *G. candidum*, and reduced green and blue mold of mandarin orange and grapefruit.

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Antagonistic effect of some saprophytic bacteria to *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli*

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Abstract

The biological control activity of several saprophytic bacteria against the bean pathogens *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* was studied in vitro and by seed inoculation of snap bean. Investigated saprophytic bacteria showed different antagonistic effect to snap bean pathogens in vitro. The intensity of antagonism, represented by a clear zone around the paper disc, varied from weak to very strong, regarding the combination of the saprophyte and pathogen strain. No significant antagonistic effect was observed on snap been seed in both treatments 7 days after inoculation.

Introduction

Pseudomonas syringae pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* are the most important pathogens of snap bean (*Phaseolus vulgaris*) in Yugoslavia (Arsenijevic and Balaz 1984, Arsenijevic et al. 1985, Obradovic 1990). In seasons with favorable climatic conditions, they cause great yield losses in bean. *Pseudomonas syringae* pv. *phaseolicola*, causal agent of halo-blight, prevails in the spring and early summer, causing losses in the green pod production for fresh market. Symptoms of common blight, caused by *X. campestris* pv. *phaseoli*, usually appears later in the season, resulting in plant defoliation and infection of seed. The majority of snap bean varieties grown in Yugoslavia are susceptible to both pathogens (Balaz 1990, Obradovic 1990). Control measures include using a three-year crop rotation, incorporating bean debris into the soil to reduce survival of inoculum, planting of disease-free seed, and chemical protection. Foliar application of some copper-based pesticides provided satisfactory control of common blight pathogen in Yugoslavia (Balaz 1991). As an additional approach, we investigated the use of biological agents for control of these bacterial diseases. Investigation was based on the estimation of antagonistic effect of some saprophytic bacteria against *P. syringae* pv. *phaseolicola* and *X. campestris* pv. *phaseoli*. The first results of our study are presented in this paper.

Materials and Methods

The antagonistic effect of nine saprophytic bacteria isolated from various hosts (Table 1) against strains of *P. syringae* pv. *phaseolicola* and *X. campestris* pv. *phaseoli* was evaluated. All strains were from Yugoslavia except B-1469 isolated provided by NCAIM Hungary. A modification of the filter-paper-disc method of Teliz-Ortiz and Burkholder (1960) was used for in vitro testing. Cooled, molten potato dextrose agar (PDA) was inoculated by adding a

loopful of the pathogen suspension (approx. 10^8 CFU/ml) and than poured into a Petri plate (9 cm). After the agar had solidified, two filter-paper discs dipped into suspension of the saprophyte strain (approx. 10^8 CFU/ml) were placed on the agar surface. As a negative control we used filter-paper discs dipped into sterile water. After incubation for 72 h at 26 °C, inhibition of the pathogen growth around the paper disc was estimated by measuring the distance between the edge of the disc and colony growth of the pathogen.

Table 1. Strains of saprophytic[Y] and bean-pathogenic[Z] bacteria used

Strain		Host plant
Ht-1/1	*Pseudomonas* sp.[Y]	*Hibiscus trionum* L.
L-69/1	*Pseudomonas* sp.[Y]	*Allium cepa* L.
B-1469	*Pseudomonas fluorescens*[Y]	unknown
Fo-9	*Bacillus* sp.[Y]	*Forsythia intermedia* Zab.
Hr-1/1	*Bacillus* sp.[Y]	*Quercus rubra* L.
Eh-20/1	*Erwinia herbicola*[Y]	unknown
Mk-19	Not determined[Y]	*Papaver somniferum* L.
Ks-464	Not determined[Y]	*Pyrus communis* L.
Ru-11	Not determined[Y]	*Rosa* sp.
SP-17	*P. syringae* pv. *phaseolicola*[Z]	*Phaseolus vulgaris* L.
SP-31	*X. campestris* pv. *phaseoli*[Z]	*Phaseolus vulgaris* L.

The antagonistic activity of saprophytic strains was evaluated on seeds by dipping surface sterilized certified seed of snap bean cv. Palanacka rana first into the suspension of the pathogen and then into the suspension of the saprophyte for 3 h each, and vice versa in the second treatment. Samples of 50 treated seeds were placed on sterile, wet filter paper in Petri plates (20 cm) and incubated at 26 °C. Symptoms of bacterial infection were observed 7 days after inoculation. As a control, three seed samples were treated only with a suspension of the pathogen, suspension of the saprophyte, and sterile water, respectively. In both plate and seed experiments, bacterial cultures were grown on nutrient agar slants for 24 h and suspended in sterile water prior to use.

Results

Investigated saprophytic bacteria showed different antagonistic effect to snap bean pathogens in vitro, on PDA, after 72 h (Table 2). The intensity of antagonism, represented by width of a clear zone surrounding the paper disc, varied from weak to very strong, regarding the combination of the saprophyte and pathogen strain (Fig. 1). No in vitro antagonistic effects against *P. syringae* pv. *phaseolicola* were observed in the case of four investigated saprophytic strains, while the other four strains showed weak antagonism (Table 2). Strain Ru-11 isolated from *Rosa* sp. had strong antagonistic activity and inhibited the pathogen growth in the zone of 16 mm around the disc (Table 2). In contrast, all strains showed some level of antagonism in vitro toward *X. campestris* pv. *phaseoli*. Intensity of antagonism varied from weak to very strong (Table 2). Four strains (B-1469, Fo-9, Eh-20/1, Ru-11), isolated from different hosts, inhibited the growth of *X. campestris* pv. *phaseoli* in the zone of over 20 mm around the disc.

In seed tests, no antagonistic effect was observed on snap been against either disease seven days after antagonist inoculation. All seed inoculated with pathogens showed symptoms of bacterial infection. Seed treated only with saprophytic bacteria or with sterile water did not have symptoms.

Table 2. In vitro antagonism of bean pathogenic bacteria by saprophytic strains[z]

	Pathogens	
	P. syringae pv. *phaseolicola*	*X. campestris* pv. *phaseoli*
Saprophyte strain		
Ht-1/1	+	+
L-69/1	+	+
B-1469	+	++++
Fo-9	-	++++
Hr-1/1	-	++
Eh-20/1	+	++++
Mk-19	-	++
Ks-464	-	++
Ru-11	+++	++++
Control	-	-

[z] Antagonistic effect: none '-', weak '+' (width of inhibited growth area 1-5 mm), medium '++' (6-10 mm), strong '+++' (11-20 mm), very strong '++++' (over 20 mm).

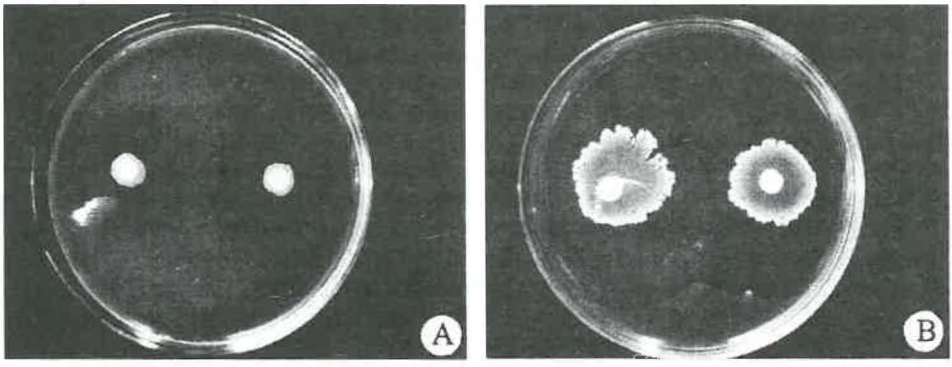

Fig. 1. The inhibitory effect of saprophytic bacteria on in vitro growth of *P. syringae* pv. *phaseolicola* and *X. campestris* pv. *phaseoli*. A: *Rosa* strain, Ru-11, strong antagonism to *P. syringae* pv. *phaseolicola* and B: *Forsythia* strain, Fo-9, very strong antagonism to *X. campestris* pv. *phaseoli*.

Discussion

Biological control of plant diseases by microbial agents has been intensively investigated during the past few years (Arsenijevic et al. 1994, Campbell 1989, El-Goorani and Hassanein 1991, Kearns and Hale 1995, 1996, Liao 1989). We investigated the utility of saprophytic bacteria isolated from various hosts as a biocontrol agents for bean diseases caused by pathogenic bacteria.

The strains studied differed in their ability to inhibit growth of *P. syringae* pv. *phaseolicola* and *X. campestris* pv. *phaseoli* in vitro and this may be due to the production of an antibacterial compound(s). Strong antagonism against bean halo blight pathogen was observed with one saprophytic strain isolated from *Rosa* sp. and very strong antagonism against bean common blight pathogen was observed with four strains belonging to different genera and originating from various hosts. No antagonistic effect was observed with seed inoculations. Unsuitable methodology could be one of the reasons for such result. Therefore, more research should be done on characterization of potential antagonists, determination of their antibacterial products and their application for pathogen control in plants. Using such products for the control of seed-transmitted pathogenic bacteria would be of great importance.

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Loss of *Agrobacterium tumefaciens* tumor inducing plasmid in solarized soil

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Abstract

One hundred and twenty-two rifampicin resistant *Agrobacterium* isolates were recovered from solarized soil that had been inoculated with a marked strain of *A. tumefaciens* before the treatment. Twelve isolates were nonpathogenic, but were similar to the inoculated strain in their biochemical characteristics and total DNA fingerprints. Plasmid profiles showed that they lacked the tumor inducing plasmid but retained another large plasmid of the same size as the cryptic plasmid of the inoculated marked strain. Preliminary data support the hypothesis that solarization can cure soil resident agrobacteria of Ti plasmid.

Introduction

The ability of *Agrobacterium tumefaciens* to induce crown gall disease on many host plants is due to the presence in the bacterial cells of a large plasmid (200 to 400 Kb) named pTi for tumor inducing plasmid.

During the infection process, a specific 24 Kb sequence of pTi, the T-DNA, is transferred from the virulent bacteria to the host plant. T-DNA transfer is regulated by virulence genes (*vir*) on pTi. The transferred T-DNA sequence is integrated in the host plant genome and its expression produces enzymes involved in the auxin and cytokinin synthesis. These hormones induce an abnormal proliferation of plant cells and subsequently the development of galls on the crown, roots, and in some cases on aerial parts of host plants. Crown gall disease causes serious losses to nurseries because affected fruit-trees and ornamental plants are unsaleable. The only effective method to control disease is the treatment of the plant roots, before transplanting, with a suspension of *A. radiobacter* strain K84 (New and Kerr 1972); however, some natural populations of *A. tumefaciens* are unaffected by the antagonist.

Solarization consists of covering the soil during the warmest period of the year with a plastic tarp. The high soil temperature together with physical, chemical and biological modifications occurring under the tarp during the treatment, determine in many cases a strong reduction of soilborne plant pathogens populations (Katan 1991).

Solarization trials were carried out to determine the efficacy of the treatment in reducing *A. tumefaciens* populations in soil (Raio et al. 1997). It is known that in vitro treatment of some *A. tumefaciens* strains at 37 °C initiates loss of the Ti plasmid (Hamilton and Fall 1971), thus it has been hypothesized that the high temperature of soil during solarization could contribute to cure agrobacteria of pTi. The objective of this work was to check this hypothesis and determine if the mutation to avirulent pTi plasmidless strains could be a second way to control tumorigenic agrobacteria in solarized soil.

Materials and Methods

Solarization trials were performed in soil that was artificially inoculated with the rifampicin resistant *A. tumefaciens* strain B49c. After one month of treatment, the marked strain was no longer detectable by traditional dilution plating method, but a residual population was recovered by an enrichment technique (Raio et al. 1997).

Six hundred rifampicin resistant isolates from untreated and 122 isolates from enriched solarized soil were tested with pinF*vir*AB, *vir*D2 and *tms*I-*tmr* pTi probes by colony hybridization. Probes labelling was performed by digoxigenin nonradioactive system (Martin et al. 1990) according to (Boehringer Mannheim Co.) manufacturer's procedure. All isolates were also tested for pathogenicity by inoculation on tomato and datura.

Isolates that did not hybridize with all the three probes were further characterized for biochemical and physiological features by Kerr and Panagopoulos (1977) biotyping tests. Plasmid profiles were prepared. Total plasmid DNA was isolated by the alkaline lysis method (Sambrook et al. 1989), electrophoresed in a 0.5% agarose gel, stained with ethidium bromide and photographed under UV light. A total DNA fingerprint was prepared. DNA extraction and digestion were carried out according to Sambrook et al. (1989). *Bgl*I and *Eco*RI restriction enzymes were used for DNA digestion.

Results

All the isolates from the nonsolarized control plots hybridized with the three probes and were pathogenic to tomato and datura. Out of 122 isolates from the enriched solarized soil, one hundred and eight hybridized with all the probes and were pathogenic, two (isolates 192 and 218) hybridized with two probes only (pinF*vir*AB, and *tms*I-*tmr*) and retained their pathogenicity, while twelve isolates did not hybridize with any probe and did not induce tumors in tomato and datura. The twelve avirulent putative mutants were isolated from different plots and from soil samples taken at different times during solarization.

The two isolates that hybridized with two of the three probes showed the same metabolic characteristics of the inoculated marked strain B49c. The twelve strains that did not hybridize and were nonpathogenic showed a metabolic pattern similar to B49c, differing only for litmus milk reaction, alkali production from malonic and mucic acid, and growth at 35 °C. Metabolic profiles are reported in Table 1.

Strains 192 and 218 had the same plasmid profile as B49c which contains pTi and another large cryptic plasmid, while the nonpathogenic isolates retained only one plasmid of size similar to B49c cryptic plasmid. Plasmid profiles are showed in Fig. 1.

DNA fingerprints obtained from complete digestion of total cellular DNA were the same for the twelve putative mutants strains and were very similar to the patterns of strains 192, 218 and to B49c, that showed some differential bands. DNA profiles after digestion with *Bgl*I restriction enzyme are shown in Fig. 2.

Table 1. Metabolic profiles of rifampicin resistant strains isolated from solarized soil.

	Strains														
Tests	2	143	154	192	218	S10	S11	S13	S14	S15	S16	S17	S18	S19	B49 cR+
Acid from: erithritol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Litmus milk reaction	alk.	alk.	alk.	acid	acid	alk.	alk.	alk.	alk.	alk.	alk.	alk.	alk.	alk.	acid
Growth in NaCl broth (%) 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on Fe ammonium citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alkali from: malonic acid	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+
Propionic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Music acid	+/-	+/-	+/-	+	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+
Growth at 35 °C	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-

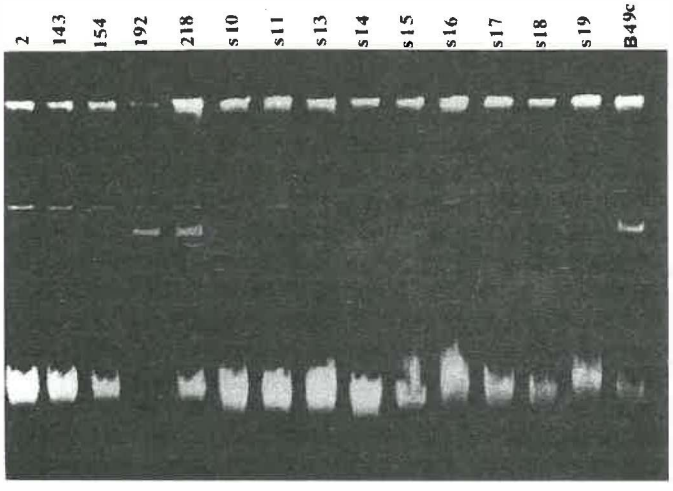

Fig. 1. Agarose gel electrophoresis of plasmid DNA

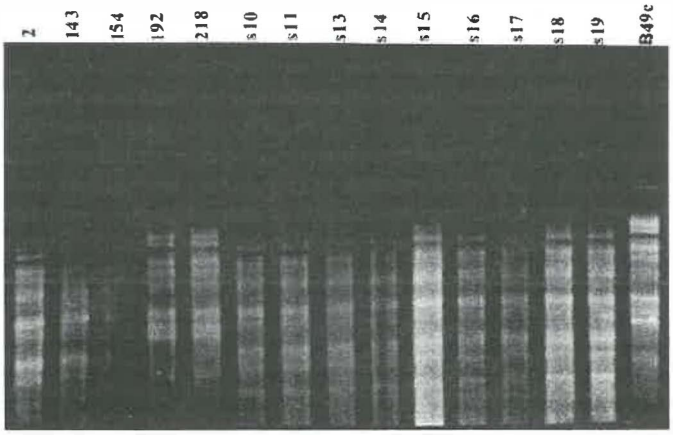

Fig. 2. Agarose gel electrophoresis of total DNA after digestion with BglI.

Discussion

Tumorigenic agrobacteria can be converted efficiently to stable avirulent derivatives by growing bacterial cultures at 32 to 37 °C. The loss of virulence can be due to the loss of the whole Ti plasmid or to a deletion of pTi sequences involved in tumorigenicity (Hamilton and Fall 1971, Lin and Kado 1977).

During solarization, temperatures higher than 35 °C were recorded in a soil artificially inoculated with the marked *A. tumefaciens* strain B49c (Raio et al. 1997).

Putative Ti plasmid-less mutants of the marked strain were isolated only from solarized soil. In contrast, all the isolates from the untreated plots were identical to B49c. About 10% of the isolates from solarized soil were avirulent and retained only one plasmid similar to the cryptic plasmid of the inoculated marked strain. Even though they had the same metabolic, biochemical and molecular characteristics, they were isolated from different plots and at different sampling times. This suggests that they were not clones of one single mutant of *Agrobacterium* cell. However, they differed from the inoculated marked strain in certain respects. The ability of the avirulent putative mutants to grow at 35 °C could be associated to the loss of pTi. Infact, Watson et al. (1975), reported that Ti plasmidless mutants of *A. tumefaciens* show an increased resistance to the high temperatures. The few differential bands observed within the digested total DNA profile of B49c, could be of pTi origin. Bouzar and Moore (1987) proved that the profiles of restriction enzymes fragments obtained from digestion of total cellular DNA are strain specific and that the presence of extrachromosomal plasmid DNA does not appreciably modify the cleavage patterns.

Preliminary data reported here supports the hypothesis that solarization can induce pTi curing of agrobacteria and that this was due to the high temperature reached during the treatment. Additional analysis is needed to determine the relatedness between the putative mutants and the inoculated strain, the frequency and repeatability of mutation in soil, and its ecological relevance.

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In vitro action of plant extracts on *Botrytis* spp.

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Abstract

The in vitro effect of extracts from *Berberis vulgaris* and *Chelidonium majus* was studied against plant pathogenic *Botrytis cinerea* Pers. isolated from species of genera *Dahlia, Rosa, Dianthus* and *Cactus, B. paeoniae* Oudem. isolated from *Paeonia* spp., and *B. tulipae* Lind. isolated from *Tulipa gesneriana.* We evaluated in vitro the effect of total extracts from *B. vulgaris* and *C. majus* on fungal growth, sporulation, and sclerotia formation. Plant extracts from *B. vulgaris* containing 1% alkaloids, and extracts from *C. majus* containing 0.25% alkaloids were added to Czapek agar at alkaloid concentrations of 25 to 250 µg/ml. Extract from both plants had increasingly inhibitory activity against all fungi with increasing alkaloid concentration.

Introduction

Plant extracts from some cormophyte species exhibit antimicrobial action (Anonymous 1968, Ionescu-Stoian and Savopol 1977, Paun et al. 1986-1988). *Botrytis* spp. are among the most problematic fungal pathogens in agriculture and horticulture worldwide. Several approaches have been taken to control *Botrytis* including fungicides and biological control. Recently, plant extracts have been shown to have some inhibitory activity. Extracts from *Chelidonium majus* L. and *Pastinaca sativa* L. were highly active in vitro against grapevine isolates of *B. cinerea* reducing pathogen growth by 90% (Jiratko and Vesela 1992). Prior application of extracts from *Petroselinum hortense* Hoffm. and *P. sativa* completely inhibited growth of *B. cinerea* on detached haricot bean leaves, on the growing tip of *Lycopersicum esculentum* Mill., and on *Cinnamomum* spp. when pathogen inoculum was applied 72 h after plant extracts (Jiratko 1994). Compound BC-1000 from grapefruit seed extract at a concentration of 1,500 ppm had inhibitory activity against grapevine isolates of *B. cinerea* comparable to the fungicides vinclozolin and benomyl (Esterio et al. 1992). Total extracts from *Berberis vulgaris* and *C. majus* have antibiotic activity on a great number of pathogens (Craciun et al. 1976, Gheorghiu et al. 1969, Pârvu 1993). The active substances of these plants are berberin alkaloids and chelidonin, respectively (Paun et al. 1986-1988, Tamas et al. 1987). Chelidonin occurs naturally at concentrations of 0.2 to 1.4% in roots and 0.012 to 0.8% in vegetative underground organs of *C. majus* (Gheorghiu et al. 1969). Activity of total alkaloids from *C. majus* has been tested on pathogenic bacteria from genera *Staphylococcus, Streptococcus, Escherichi, Pseudomonas* and *Candida* fungus pathogenic to man (Gheorghiu et al. 1969). In this study, we investigated the optimal dosage of total extracts from *B. vulgaris* and *C. majus* for inhibition of *Botrytis* spp. isolated from ornamental plants.

Materials and Methods

Pathogenic fungi used were *B. cinerea* Pers., *B. paeoniae* Oudem., and *B. tulipae* Lind. *B. cinerea* was isolated from species of genera *Dahlia, Rosa, Dianthus* and *Cactus*. Each isolate was studied in separate experiments. Total extracts from *B. vulgaris* and *C. majus* contained 1 and 0.25% alkaloids, respectively, and were obtained by the volumetric method from plants collected and processed according to standard methods (Anonymous 1968, Gheorghiu et al. 1969, Paun et al. 1986-1988). To obtain the total extract from the *C. majus* species we collected the airy plant organs, called 'Herba Chelidonii'. The airy parts were dried after collecting, the were macerated, and were introduced in an 70% ethyl alcohol solution. After a day, the total extract was collected. In our experiments we used total extract without ethylic alcohol. The total extract from *B. vulgaris* was obtained in the same conditions, but for this we used the bark of stem.

Extracts were added to Czapek agar (pH 7.2) after autoclaving to give alkaloid concentrations ranging from 25 to 250 µg/ml. Conidial suspensions (10^{-5}/ml) of *Botrytis* were placed in the center of plates with extracts. Plates were incubated 12 days at 22 °C, the optimal growth temperature for the fungi. Each treatment consisted of five replicate plates. Antifungal activity was evaluated based on colony diameter (mm), sporulation and sclerotia formation, compared with growth on nonamended media. In *B. vulgaris* extract the predominant alkaloid is berberine and in *C. majus* chelidonine. These alkaloids are active substances of total extracts. Data have been treated by analysis of variance (ANOVA).

Results and Discussion

Total extracts from *C. majus* (Table 1) and *B. vulgaris* (Table 2) had antifungal activity against *B. cinerea, B. paeoniae,* and *B. tulipae*. Increasing alkaloid concentrations corresponded with increased inhibition. The highest active concentration of alkaloids varied with fungus and with plant origin of the extract. Total inhibition was observed at alkaloid concentrations of 250 µg/ml. The antifungal properties of plant extracts against *Botrytis* species can serve as starting point in establishing some elements of biological control.

Table 1. In vitro activity of *Chelidonium majus* extract against *Botrytis* spp.

Fungus	Alkaloid conc. (µg/ml)	Colony diam.[Y] (mm)	Sporulation[Z] %	Sclerotia[Z]	Inhibition
Botrytis cinerea	250	0	-	-	100.0
	225	5.0	-	-	92.8
	200	10.0	-	-	85.7
	175	18.0	-	-	74.2
	150	25.0	-	-	64.2
	100	45.0	-	-	35.7
	25	65.0	+	++	7.1
	0	70.0	++	++++	-
Botrytis paeoniae	250	0	-	-	100.0
	225	0	-	-	100.0
	200	6.0	-	-	91.4
	0	70.0	+	++++	-
Botrytis tulipae	250	0	-	-	100.0
	225	0	-	-	100.0
	200	8.1	-	-	88.4
	0	70.0	+++	++	-

[Y] Within a fungus, growth was significantly ($P < 0.05$) reduced by all alkaloid concentrations compared with the nonamended control.

[Z] For sporulation and sclerotial formation, respectively: '-' is absent; '+' is poor, 1-20 sclerotia/plate; '++' is moderate, 21-40 sclerotia/plate; '+++' is dense, 41-60 sclerotia/plate; '++++' is abundant, >60 sclerotia/plate.

Table 2. In vitro activity of *Berberis vulgaris* extract against *Botrytis* spp.[Z]

Fungus	Alkaloid conc. (µg/ml)	Colony diam. (mm)	Sporulation %	Sclerotia	Inhibition
Botrytis cinerea	250	0	-	-	100.0
	225	4.0	-	-	94.2
	200	8.1	-	-	88.5
	0	70.0	++	++++	-
Botrytis paeoniae	250	0	-	-	100.0
	225	0	-	-	100.0
	200	7.0	-	-	90.0
	0	70.0	+	++++	-
Botrytis tulipae	250	0	-	-	100.0
	225	6.0	-	-	91.4
	200	10.0	-	-	85.7
	175	18.0	-	-	74.2
	150	23.0	-	-	67.1
	100	45.0	+	-	35.6
	25	60.0	++	+	14.2
	0	70.0	+++	++	-

[Z] See legend for Table 1.

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Biological control of *Pythium* in greenhouse cucumber

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Abstract

When growing greenhouse cucumber in compost problems with soilborne disease will turn up after few seasons, especially when rotation is not used or possible in greenhouses. A common disease is *Pythium* root rot (black leg) caused by *P. ultimum*. To prevent this disease different prevention methods have to be used. In a Danish project with organically grown cucumber an investigation of the following methods are carried out: 1) Biological control using the commercial fungal agent Polyversum containing oospores of the antagonistic fungus *Pythium oligandrum*. 2) Systemically induced resistance to diseases in plants using different treatments, which increase the resistance of the plants to attack of *Pythium*. At the same time experiments with a method for registration of induced resistance as changes in the chemical components of the plant are carried out. 3) Solarization, a non-hazardous method of soil disinfection, where temperatures at 40-50 °C are obtained from solar heating. These temperatures are lethal or suppressive for *Pythium*, but many other microorganisms including fungi and bacteria will survive. This shift in the microbial pattern can increase activity of antagonists and other soil microorganisms.

Biological control. Soil and primed seeds were treated with oospores of *P. oligandrum* before sowing. Two weeks later half of the seedlings were inoculated with *P. ultimum*. The experiments showed that the soil treatment has no effect and that the seed treatment only has a little effect and not always.

Induced resistance. Resistance was induced with the following treatments; a) seed treatment with the antagonistic microorganism *Pythium oligandrum* (Biopreparate Polversum), b) watering of seedlings with K_2PO_4 and, c) watering of seedling with hot water. Half of the plants were inoculated with *P. ultimum* two days later. Only temporary results are available yet.

Solarization. Greenhouse experiments were carried out in 1995-1996 testing solarization as a method to control root rot caused by *Pythium ultimum* in organically grwon cucumber. Solarization was carried out as follows: Moist soil was covered with transparent, 0.05 mm polyethylene (PE), for 4 or 8 weeks in June/July. Dry soil without PE served as control. Results in 1995 and 1996 showed soil temperatures above 40 °C and a reduction of *Pythium* propagules after 4 weeks' treatment. Inoculum density and temperature are measured in the upper 10 cm soil. After the solarization period in 1996 cucumbers were planted and examined 13 weeks later. Disease was considerably reduced in solarized soil compared to non-solarized soil.

These three methods give new prospects of controlling soil-borne plant pathogens in greenhouses, possibly combined with resistant varieties and methods of cultivation.

Effect of trichothecin on metabolic processes in wheat leaves infected by stem rust and powdery mildew

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Abstract

Trichothecin is antibiotic produced by *Trichothecium roseum*. We have studied the fungitoxic effect of trichothecin on the isogenic lines of wheat (*Triticum aestivum* L.) differing in resistance to stem rust and powdery mildew. Spraying of plants with the antibiotic fully prevented them from these diseases. Both roots and leaves responded to trichothecin applied to roots with increases in reate of protein synthesis, in activities of oxydative enzymes, phenol content, leaf photosynthesis and respiration. Alterations in metabolic processes induced by trichothecin increased the resistance of wheat seedling to obligatory pathogens.
Trichothecin treatment led also to alterations in isozymes of peroxydase; these changes were observed for components with small and high electrophoretic mobility. The relationship between metabolic changes caused by trichothecin and plant resistance to stem rust and powdery mildew is discussed.

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