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Influence of A-Biotic and Biotic Factors on Biocontrol Agents

editors:

Yigal Elad, Jürgen Köhl & Dani Shtienberg

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Working Group
"Biological Control of Fungal and Bacterial Plant Pathogens

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Influence of A-Biotic and Biotic Factors on Biocontrol Agents

at

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Edited by
Yigal Elad, Jürgen Köhl and Dani Shtienberg

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PREFACE

There is increasing interest in biocontrol of plant pathogens. The number of people who study aspects of the interactions between plant pathogens and bacteria, fungi, phages or viruses and the number of publications increased steadily with time. In some cases the search for biocontrol agents lead to implementation under field conditions and commercial development of biological preparations for control. However, The number of commercial preparations is yet small and much more is expected from us before significant phytopathological problems could be solved by biological means.

One of the reasons that preclude implementation of biocontrol agents on a large scale in commercial production is the large variability in control efficacy. In some cases the biocontrol agents are as effective as chemical products, but in other they are less effective and sometimes they are not effective at all. The assumption underlying this meeting was that one of the obstacles on the way of reliable biocontrol under field conditions is the interference by biotic or a-biotic factors. These factors affect the activity, survival and interaction of the biocontrol agents with the plant pathogens or with plants. In the meeting we presented our knowledge in this regard and discussed the possibilities to overcome deleterious effects on the implementation of biocontrol of plant diseases.

It is a great pleasure to thank all persons that contributed to the buildup and success of the meeting. Special thanks to Jürgen Köhl (PRI, Wageningen, The Netherlands), Nafiz Delen (Ege University, Turkey), Dani Shtienberg who took care of perfect presentation, Fazilet Vardar Sukan (EBILTEM, Ege University, Turkey), Yildiz Hepileri Gurgun (Dalya Turizm) and their teams and to the Organizing, Scientific and International Advisory Committees.

I wish all of us fruitful, interesting and enjoyable meetings also in the future.

Yigal Elad, Convenor
IOBC/WPRS WG on "Biological control of Fungal and Bacterial Plant Pathogens"
July 2002

As the editors of the present volume, we wish to thank all the contributors for summarizing their works. The contributions present valuable information for the science and implementation of biocontrol of plant diseases. The contributors were very cooperative with the editors and facilitated rapid completion of the volume.

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Previous meetings of the phytopathogens working group:
I- New Approaches in Biological Control of Soil-borne Diseases, Copenhagen, July, 1991.
II- Biological Control of Foliar and Post-Harvest Diseases, Wageningen, The Netherlands, Dec., 1992.
III- Biocontrol of Sclerotium-Forming Pathogens, Wellesbourne, UK, Dec., 1994.
IV- Biological and Integrated Control of Root Diseases in Soilless Cultures, Dijon, France, Sept,. 1995.
V- Role of Molecular Methods in Disease Control, Belemont Zurich, Switzerland, 1996.
Session on Biocontrol of Plant Diseases, 7th ICPP Congress, Edinburgh, August 1998.
VI. Biocontrol agents modes of action and integration with other means of control, Sevilla, Spain, Nov.-Dec.2000.

Present meeting: Influence of A-Biotic and Biotic Factors on Biocontrol Agents

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Chair persons: Leszek Orlikowski and Hatice Ozaktan

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Chair persons: Aleid Dik and Figen Yildiz

Session 5: Soil factors and biocontrol
Chair persons: Dan Funk Jensen and Enrique Monte

Poster group 4: Environmental factors and screening of better biocontrol agents
Chair persons: Eckhard Koch and Naresh Magan

Posters group 2: Modes of action and interactions in the plant-pathogen-biocontrol agent system
Chair persons: Monica Höfte and Haïssam Jijakli

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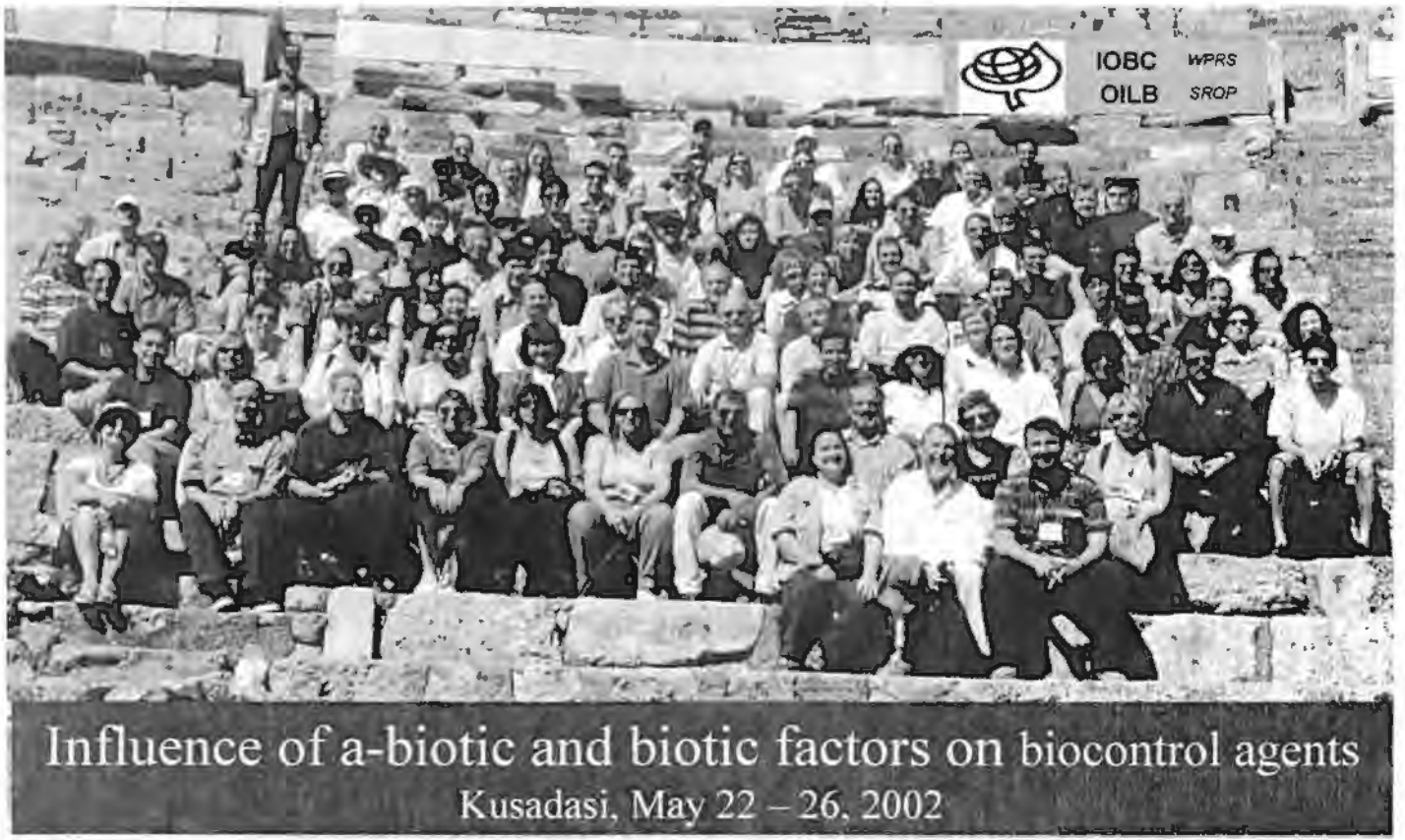
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Is it possible to cope with variability of biocontrol?

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Abstract: Implementation of biocontrol on a large scale is still limited and the number of pathosystems where it is commercially used is not high. One of the major reasons that hinder the implementation of biocontrol on a large scale is the variability and inconsistency of biocontrol activity. Several lines of evidence now suggest that variability of biocontrol results (among other things) from the sensitivity of the biocontrol agents to environmental influences. One of the ways to overcome the inability of most of the biocontrol agents to be effective under diverse environmental conditions is to integrate them with chemical fungicides; Integration should be done according to ecological requirments of the biocontrol agents. Introduction of two or more biocontrol agents to the phyllosphere, assuming that each one of them has different ecological requirements, may result in increased control consistency as well. Another approach to overcome the variability of biocontrol is to use biocontrol agent(s) with several modes of actions. Implementation of one (or more) of these approached is expected to lower the variability and increase the consistency of biocontrol. This, in return, will reduce the risk of uncontrolled epidemics and increase the confidence of growers in using this non-chemical control measure on a large scale.

Key words: biological control, *Botrytis* cinerea, integrated pest management

Introduction

Biocontrol of plant pathogens was studied for decades by numerous researchers all over the world. The successes are indisputable and biocontrol agents are used commercially with appreciable success in many countries. Despite this success problems still exist. Implementation of biocontrol on a large scale is still limited and the number of pathosystems where it is commercially used is not high. Perspectives for the future are not encouraging, as the situation is not expected to change. One of the major reasons that hinder the implementation of biocontrol on a large scale is the variability and inconsistency of control efficacy. Whereas in some cases the efficacy of biocontrol is adequate and as good as that achieved by chemical fungicide, in other cases the efficacy of biocontrol is inferior to that of the chemical alternative. Moreover, sometimes the biocontrol agents are not effective at all (Elad, 1990). The problem is that it is not possible to know in advance, what will be the efficacy of the biocontrol measures. Thus, growers that attempt to minimize risks from uncontrolled epidemics avoid of using biocontrol.

To illustrate the inconsistency and variability of biocontrol, example for biocontrol of *Botrytis cinerea*, the causal agent of gray mold, is be given. Gray mold epidemics are a serious problem in vegetable greenhouses and are often the reason for finishing a crop earlier than planned. A commercial preparation developed from an isolate of *Trichoderma harzianum* T39 (TRICHODEX, Makhteshim Agan Ltd., Be'er Sheva, Israel) was registered for agricultural use in Israel and other countries. Analysis of 64 experiments carried out in nonheated vegetable greenhouses during 1988-1993 revealed that *T. harzianum* T39, when applied alone, was as effective as the standard chemical treatment in 70% of the trials. In

some experiments (20%), however, disease suppression achieved by *T. harzianum* T39 was significantly inferior to that of the standard chemical treatment and in a few experiments (10%) *T. harzianum* T39 efficacy was insufficient (Shtienberg & Elad, 1996).

Inconsistency and variability in the efficacy of biocontrol in commercial situations may have several causes. It was suggested that environmental conditions, which are not fully controlled (or not controlled at all), in commercial production may influence the survival, establishment in the phyllosphere and activity of the biocontrol agents. Under commercial conditions, the phylloplane is subjected to fluctuating temperatures, relative humidities, surface wetness, gases and air movement. These conditions affect the phyllosphere microflora (including the biocontrol agents) directly, or may have an indirect affect by modifying the characteristics of the host plant. In order to improve consistency of biocontrol and reduce its variability, attepts should be made to overcome the sensitivity of the biosontrol agents to the flactuating environment. This may be done by selecting biocontrol agents that their eclogocal requirments encompass those of the pathogen (Köhl & Molhoek, 2001) or by applying the biocontrol agents only when they are expected to be effective.

Considering the Ecological Requirments of the Biocontrol Agents

Integration of biological and chemicial mesures

One of the ways to overcome the inability of most of biocontrol agents to be effective under diverse environmental conditions is to integrate them with chemical fungicides. As the biocontrol agents are living organism that are affected by the environment (as do the pathogens), integration of biocontrol agents with chemical fungicides should be done according to the ecological requirments of both the pathogen and the biocontrol agent. The ecological requirements of *B. cinerea* and *T. harzianum* T39 differ to some extent. In general, gray mold epidemics occur in cool and humid conditions, which favor infection and may also predispose the host to susceptibility. *T. harzianum* T39, on the other hand, provides the most effective control at high temperatures and under conditions of low vapor pressure deficit, but not when the air is saturated or when dew is deposited on the foliage. Comparing the ecological requirements of *B. cinerea* and *T. harzianum* T39 suggests that this biocontrol agent would be most effective when conditions are suboptimal for *Botrytis* development. When conditions are highly conducive to the pathogen, the efficacy of *T. harzianum* T39 is expected to be low (Shtienberg & Elad, 1996).

A conceptual framework for integration of *T. hartzianum* T39 and chemical fungicides was developed based on the ecological requirments of the biocontrol agent and the epidemiology of the pathogen (Shtienberg & Elad, 1996). The integrated strategy (named BOTMAN, Botrytis manager) is implemented as follows: when slow or no disease progress is expected, no spraying is needed; on the other hand, when an outbreak of epidemics is expected, use of a chemical fungicide is recommended; in all other cases, application of *T. harzianum* T39 is recommended. The integrated strategy was compared with weekly application of fungicide in 15 experiments conducted over 4 years in greenhouse-grown tomatoes and cucumbers. Disease reduction in plots treated according to BOTMAN did not differ significantly from that achieved in plots treated only with chemical fungicides, in all 15 experiments. The number of fungicide sprays in the integrated strategy ranged from 2-7 (mean 4.2) compared to 7-13 (mean 10.5) in the fungicide treatment. The integrated strategy averaged additional 5.9 sprays of *T. harzianum* T39. Thus, considering the ecological requirments of the biocontrol agent while integrating it with chemical fungicides may reduce the variability of biocontrol without affecting control efficacy.

Integration of several biocontrol agents with complimentary ecological requirments

Another way to overcome the inability of most biocontrol agents to be effective under diverse environmental conditions is to introduce more than one biocontrol agent to the phyllosphere, assuming that they have complementary ecological requirements. This may facilitate control efficacy of single organism under diverse conditions and result in increased control consistency. To test this possibility, the combined efficacy of two biocontrol agents, a yeast (*Pichia guilermondii* isolate Y2) and a bacterium (*Bacillus mycoides* isolate B16) was studied. The host in these experiments was strawberry and the pathogen was *B. cinerea*. The ecological requirements of the two microorganisms differed when conditions were extreme: whereas *P. guilermondii* reduced *B. cinerea* spore germination at temperatures lower than 25°C, *B. mycoides* was more effective above 25°C.

Effect of the biocontrol agents on suppression of *B. cinerea* spore germination and gray mold lesion formation were studied under a wide variety of temperature and relative humidity regimes. Both biocontrol agents, when applied separately, were highly effective at suppressing *B. cinerea* under a range of environmental conditions. However, their efficacy under other regimes was insufficient (Guetsky et al., 2001). Combined application of *P. guilermondii* and *B. mycoides* resulted in adaquate suppression of *B. cinerea* under all the microclimate regimes tested. Moreover, variability and inconsistency in control efficacy in the combined treatment were low in all experiments under all temperature and relative humidity combinations. Thus, combined application of the two biocontrol agents not only resulted in improved suppression of *B. cinerea*, but also reduced the variability of disease control (Guetsky et al., 2001).

Combinibg of biocontrol agents with several, dissimilar modes of action

Another approach to overcome the variability of biocontrol is to apply biocontrol agent(s) with several modes of actions. Biocontrol with multi-mechanisms may be achieved by using one biocontrol agent exhibiting several mechanisms or by applying more than one biocontrol agent in a mixture, provided that each of them has one (or several) distinct mechanisms and assuming that each mechanism is not negatively affected by the activity of the other. As the environment may dissimilarly influence different mechanisms of control, under a certain set of conditions one mechanism may compensate for the other(s). We tested this approach using the gray mold-strawberry pathosystem and the two biocontrol agents mentioned above, *P. guilermondii* and *B. mycoides*, as a model.

Scanning electron microscopy revealed significant inhibition of *B. cinerea* conidial germination in the presence of *P. guilermondii,* whereas *B. mycoides* caused breakage and destruction of conidia. The modes of action of each of the biocontrol agents were elucidated and the relative quantitative contribution of each mechanism to suppression of *B. cinerea* was estimated. *P. guilermondii* competed with *B. cinerea* for glucose, sucrose, adenine, histidine and folic acid. Viability of the yeast cells played a crucial role in suppression of *B. cinerea* and they secreted an inhibitory compound(s) that had an acropetal effect and was not volatile. *B. mycoides* did not compete for any of the sugars, amino acids or vitamins examined in a level that would affect *B. cinerea* development. Viable cells and the compound(s) secreted by them contributed similarly to *B. cinerea* suppression. The bacteria secreted volatile and non-volatile inhibitory compounds and activated the defense systems of the host. The non-volatile compound(s) had both acropetal and basipetal effects.

The cumulative effects of several control mechanisms may be illustrated by using the results obtained for suppression of *B. cinerea* conidial germination. In the control treatment, 88 to 96% of *B. cinerea* conidia germinated. When supernatant from live cells was added,

germination decreased to 67% (for *P. guilermondii*) or to 47% (for *B. mycoides*). This decrease presumably resulted from an inhibitory compound(s) secreted by the microorganisms into the medium. Adding live cells to the system decreased *B. cinerea* germination to 28% (for *P. guilermondii*) or to 22% (for *B. mycoides*). Combining live cells of both biocontrol agents further decreased *B. cinerea* conidial germination to 8% (Guetsky et al., 2002). When both biocontrol agents were applied in a mixture, their activity reflected the sum of the biocontrol mechanisms of each biocontrol agent separately. The fact that mixture of *P. guilermondii* and *B. mycoides* resulted in additive activity, as compared to their separate application, was also demonstrated by scanning electron microscope observations that revealed severe destruction and shrinkage of *B. cinerea* conidia. The effects of the mixture were markedly more severe than that of each biocontrol agent alone (Guetsky et al., 2002).

Conclussions

Several strategies to cope with biocontrol inconsistency and variability were suggested. Combination of biocontrol agents among themselves or with chemical fungicides, have the potential to solve the problem. The purposed strategies fits in well with the concepts of integrated pest management, in which several means of disease suppression are applied concurrently. When one or more means/mechanisms are not effective, the others may compensate for the former absence. Implementation of these approached is expected to lower the variability and increase the consistency of biocontrol. This, in return, will reduce the risk of uncontrolled epidemics and increase the confidence of growers in using this non-chemical control measure on a large scale.

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Combinations of control methods against powdery mildew diseases in glasshouse-grown vegetables and ornamentals

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Abstract: Biocontrol agents, salts and other natural substances were tested for efficacy against powdery mildew in greenhouse vegetables, roses and potted plants, as stand-alone treatments or in combination or alternation with each other. For all crops, one or more compounds successfully controlled powdery mildew. When conditions were conducive for disease outbreak, integration with partially resistant cultivars improved disease control.

Key words: *Ampelomyces quisqualis*, cucumber, Kalanchoe, *Leveillula taurica*, Milsana, $NaHCO_3$, *Oidium lycopersicum*, pepper, potted plants, *Pseudozyma flocculosa*, rose, *Sphaerotheca fuliginea*, *Sphaerotheca pannosa* tomato, *Trichoderma harzianum* T39

Introduction

Powdery mildew fungi can cause severe disease problems in greenhouse-grown cucumber, pepper, tomato, roses and potted plants. Research in Applied Plant Research division Glasshouse Horticulture (PPO, formerly PBG), in collaboration with Dr. R.R. Bélanger (Université Laval, Canada), Dr. Y. Elad (The Volcani Center, Israel) and Dr. A. Ernst (Gebruder Schaette, Germany) focussed on control of powdery mildew diseases with biocontrol agents, plant extracts, salts and oils. These compounds have been tested as stand-alone treatments as well as in combination or alternation. Aim of the research was to reduce the input of chemical fungicides and the dependence on these fungicides. In this paper, we will give a brief overview of the results.

Material and Methods

Efficacy trials

Large-scale greenhouse trials were conducted in cucumber, tomato, pepper, rose and potted plants. All experiments were laid out as complete randomised blocks with 4-6 replicates and between 4 and 11 treatments. All experiments included a control treatment sprayed with water and a chemical control treatment where fungicides were applied routinely. In rose and potted plants, an untreated control was added. The biocontrol agents that were tested were:

- *Pseudozyma flocculosa* (syn. *Sporothrix flocculosa*, product Sporodex, Plant Products LTD., Canada): tested in cucumber, tomato, pepper, rose and potted plants.
- *Ampelomyces quisqualis* (AQ10, Ecogen Inc., U.S.A.): tested in cucumber, pepper and rose.
- *Verticillium lecanii*: tested in cucumber and rose.
- *Trichoderma harzianum* T39 (TRICHODEX, Makhteshim Agan LTD, Israel): tested in pepper and potted plants.

Natural compounds tested were: KH_2PO_4 (3.4 g/l), $MgSO_4$ (1.0 g/l), $MnSO_4$ (1.0 g/l),

$NaHCO_3$ (2.5–5.3 g/l) and $KHCO_3$ (8.0 g/l); All were tested in cucumber, tomato and rose. $NaHCO_3$ was also tested in pepper and potted plants. Extracts of *Reynoutria sacchalinensis* (Milsana, Gebr. Schaette, Germany) were tested in potted plants, cucumber, tomato and pepper. Oil (JMS Stylet-oil, JMS Flower Farms, Inc. FL, USA) was tested in pepper and in potted plants.

All experiments were done according to the EPPO guidelines with standard heating and ventilation set points for each crop. Disease was assessed as percentage diseased leaf area for several leaves per plant in rose, potted plants and pepper and on all leaves in tomato and cucumber. Severity per plant was calculated and averaged per plot. Disease severity records were used to calculate the Area Under the Disease Progress Curve (AUDPC). In the vegetable experiments, harvested fruits were counted and weighed at each harvesting date. AUDPC and total fruit yield per plant were analysed with analysis of variance followed by Fisher's protected LSD test at $P \leq 0.05$.

Demo trials

In the vegetable crops, large-scale demo trials were run in 2001 to assess the economic feasibility of the control methods. Not only disease and yield were considered, but also the number of applications, the time spent on applications and the amount of the substances sprayed. For each crop, one greenhouse was grown according to current practice with standard chemical fungicides on as-needed basis, and one greenhouse was grown with (combinations of) the agents mentioned above, also on as-needed basis. Nutrient solutions and climatic conditions in the greenhouses were the same. In each greenhouse, 4-8 plots of ten plants each were designated for assessment of disease and yield, as described before.

Results and discussion

Efficacy trials

In cucumber, the biocontrol agent *P. flocculosa* was effective in control of *Sphaerotheca fuliginea* in most experiments (Dik et al., 1998). In summer, this biocontrol agent should be combined with a partially resistant cultivar. The five salts tested in cucumber were not very effective (Figure 1), but when the climatic conditions are not conducive for *P. flocculosa*, alternation with $NaHCO_3$ provides a good solution since there is no direct interaction between the two.

In tomato, $NaHCO_3$ and $KHCO_3$ were the most effective of five tested salts (Figure 1). Milsana, the plant extract of *R. sacchalinensis*, gave very good control of the disease as well.

In pepper, *P. flocculosa*, *T. harzianum* T39, mineral oil and $NaHCO_3$ were tested in different combinations and application frequencies. The best results were obtained with the oil and *T. harzianum* T39 + oil, even when applied only every other week. *P. flocculosa* and $NaHCO_3$ reduced powdery mildew by approximately 50% compared with the control (Figure 2).

In roses, similar results were found for the control of *Sphaerotheca pannosa*. The salts $NaHCO_3$ and $KHCO_3$ and the biocontrol agent *P. flocculosa* gave good control and can be used in alternation (Figure 3).

In potted plants, *T. harzianum* T39 may be effective, but the current formulation results in visible residue on the plants. *P. flocculosa*, $NaHCO_3$, Milsana and oil all reduce diseases caused by the different pathogens in each crop (Figure 4). However, $NaHCO_3$ may cause some damage on the flowers when used in high concentrations.

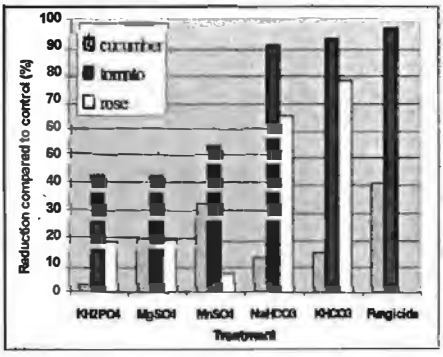

Figure 1. Efficacy of salts in cucumber, tomato and rose, expressed as reduction in disease severity compared to control with water

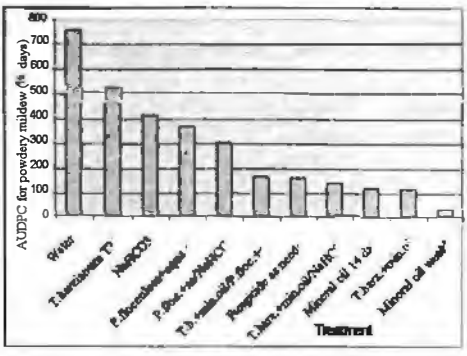

Figure 2. Effect of treatments in pepper, as stand-alone or in combination, on AUDPC

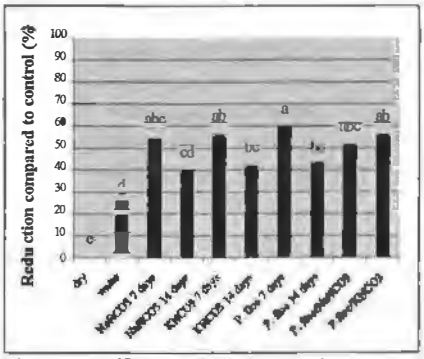

Figure 3. Efficacy of treatments in roses

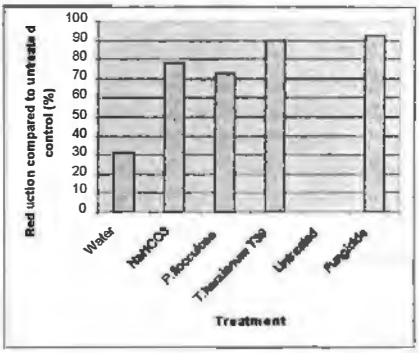

Figure 4. Efficacy of treatments in Kalanchoe

Demo trials

In the demo trials with cucumber, good control was achieved with the non-chemical treatments in the partially resistant cultivars Flamingo and Aramon, but not in the susceptible cultivar Euphoria in summer. In the partially resistant cultivars, disease was more severe in the treatment with *P. flocculosa* than in the fungicide-treated greenhouse, but yields were much higher (Table 1). This may indicate that with control agents that are 'soft' for the crop, more disease can be tolerated than with stronger agents.

In the demo trials in tomato and cucumber, disease was effectively controlled by Milsana and $NaHCO_3$. Yields were at least as good in the greenhouse with non-chemical control compared to the greenhouse in which fungicides were sprayed (Table 1).

Table 1. Area Under the disease Progress Curve (AUDPC) for powdery mildew, fruit yield per plant and total number of sprays in three demonstration trials in cucumber in 2001.

Cultivar	Treatment	AUDPC (% days)	Yield (kg plant-1)	# of sprays
Flamingo	Milsana	54.1	15.7	7
	P. flocculosa	198.6	20.4	7
	Fungicides	2.0	12.6	7
Aramon	Milsana	0.2	15.7	7
	P. flocculosa	49.3	14.5	7
	Fungicides	3.5	13.3	4
Euphoria	Milsana	683.8	5.2	6
	P. flocculosa	1035.2	4.6	6
	Milsana / *P. flocculosa*	875.4	4.6	8
	Fungicides – low frequency	198.0	6.0	6
	Fungicdes – high frequency	67.4	6.4	7

Conclusions

In conclusion, it can be stated that several control measures provide good perspective for the control of powdery mildew diseases in protected crops. Registration of these control measures will lower the dependency on fungicides for disease management and hopefully lead to a reduction in fungicide use. Integration of the effective non-chemical control measures with less susceptible cultivars will further improve control efficacy of the powdery mildew diseases.

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Colonisation of different positions in grape bunches by potential biocontrol organisms and subsequent occurrence of *Botrytis cinerea*

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Abstract: The potential of three fungal antagonists (*Gliocladium roseum, Ulocladium atrum* and *Trichoderma harzianum*) and one yeast (*Trichosporon pullulans*) to colonise different positions in grape bunches, and to reduce *Botrytis cinerea* infection, was investigated in two commercial vineyards. Colonisation by the three fungal antagonists was erratic and generally fluctuated in a season, between seasons, and between the two vineyards. Only *T. harzianum* occupied the various positions from pre-pea size to bunch closure in bunches. Occupation was better and more consistent in Chardonnay wine grape bunches grown in the wetter Stellenbosch region than in Dauphine table grape bunches grown in the drier Paarl region. This showed that two constraints, namely uneven dispersal of antagonists in the inner bunch, and unfavourable weather conditions, could hamper the successful integration of different micro-organisms exhibiting different modes of action in a disease control program.

Key words: grey mould, bunch rot

Introduction

In the Western Cape province of South Africa, grapevine is cultivated for wine and table grape production and *Botrytis cinerea*, the causal agent of grey mould, poses a menace to the producer. The pathogen follows a similar trend in local vineyards as in other parts of the world, namely to develop unnoticed early in the season, and then to cause grey mould late in the season, or during storage. For years this characteristic of the fungus has perplexed local grape producers and plant pathologists when making recommendations towards effective disease control. To overcome this problem, overseas literature on the disease is often consulted and action planned accordingly. However, it was found that overseas and local findings about the disease sometimes differ. The most prominent differences are on aspects of inoculum ecology in grape bunches, and on the infection route followed by the pathogen (Coertze & Holz, 1999; Coertze, Holz & Sadie, 2001). These differences can be ascribed to the influence exerted in local vineyards on *B. cinerea* by different sets of cultivation practices, environmental and climatical conditions.

In vineyards, the fungus is dissaminated mainly as conidia, carried by air currents and raindrops. Air currents and raindrops deposit conidia as single cells on berry surfaces. Berries are therefore not covered by masses of conidia, but carry separate conidia. The conidia do not only land on berries, but also at other positions on the vine. Each part of the vine is therefore a potential target for the fungus. Studies made with spore traps in commercial vineyards showed that amounts of conidia are high from bloom to bunch closure, and then decrease.

Estimations of viable *B. cinerea* residing naturally in bunches, showed that their amounts depicted levels occurring in the air currents. The pathogen also occurred in a constant pattern at various positions in bunches. It mostly occupied the bases of the berry and

the pedicel. The next prominent positions colonised were the rachises and laterals. It was found infrequently on the berry cheek, and the stylar end of the berries was virtually free of the pathogen.

Different techniques were used to promote symptom development. In these tests it was found that disease expression displayed the pattern showed by the inoculum deposition. It was consistently first noticed at the berry base. On some of the berries that yielded the pathogen at the base, sporulation remained confined to this position. Individual lesions seldom occurred on the berry cheek, and from this position the pathogen developed from a few berries initially. The stylar end of the berries mostly remained asymptomatic.

According to this pattern of natural occurrence in grape bunches, Botrytis bunch rot is not caused by colonisation of the pistil, and subsequent latency in the stylar end of the berry (McClellan & Hewitt, 1973). The very low amounts of *B. cinerea* occurring on the skin surface, or in the skin tissue, should also not cause berry rot. Berry rot is caused primarily by inoculum occurring at the berry base, or at the pedicel base. We also found that the amount of viable latent mycelia at the base declined gradually during the season. This indicates that *B. cinerea* bunch rot is caused primarily by conidia dispersed in bunches during early season, and that the most important inocula are those occuring superficially on the pedicel or berry bases.

In practice, disease symptoms are prominent in vineyards only after the bunch closure stage. Thus, in spite of the high amounts of inoculum occurring in bunches during early season, grey mould generally does not occur. Symptom expression only develops when host resistance is terminated by applying stress factors. This implies that the pathogen requires assistance to enable the infection cycle to run its full course, and to generate a symptom. There are strong indications that factors which may cause "weak spots" at the pedicel end of the berry, play a determining role. This explains why bunch rot mostly develop from the inner bunch and become prominent only after the bunch closure stage in vineyards. Therefore, in our efforts to control bunch rot, attempts should concentrate on the pre-bunch closure stage and on the proper protection of the pedicel-berry base zone, which is in the inner bunch.

Based on this knowledge, the protective effect of four biocontrol agents was investigated. The premise was that a combination of different organisms, each able to effectively colonise a specific position, but preferentially the bases of the pedicel/berry, had to be used in an integrated disease control programme.

Materials and methods

Spray Programmes

Unpatented isolates of *Ulocladium atrum*, *Gliocladium roseum*, *Trichoderma harzianum* and *Trichosporon pullulans* were used. All isolates were from an established laboratory and cultures had a proven record of biocontrol activity. *U. atrum* was selected for its ability to quickly occupy dead tissue in the bunch and to prevent *B. cinerea* from becoming established in, and forming conidia on it. *G. roseum* was selected to colonise the rachis, lateral and pedicel and to protect these positions from infection by *B. cinerea*. *T. harzianum* is able to grow on any of the bunch parts, and is also known for its ability to activate the inherent resistance mechanisms of the vine, thereby increasing the natural level of protection. *T. pullulans* operates only on the berry surface of ripe grapes. The fungal antagonists were applied in 1996 to 2000 in different programmes as sole agents, or in combination with the yeast, iprodione, or pyrimethanil, to bunches in commercial vineyards planted with the wine grape cultivar Chardonnay in the wetter, cooler Stellenbosch region, or the table grape cultivar Dauphine cultivated in the drier, warmer Paarl region.

***Colonisation of bunch parts by antagonists and* B. cinerea**

Bunches, collected 2 wk after each application, were left unsterile to determine surface colonisation, or were surface-sterilised to determine penetration. Presence of the fungal antagonists and of *B. cinerea,* was determined by observing bunches for fungal growth, and with isolation studies. For symptom expression, the bunches were divided in sections bearing three to seven berries on a short rachis. The sections were immersed in a paraquat solution to terminate host resistance and incubated in moist chambers to promote the development of the fungal antagonists and *B. cinerea* (Cerkauskas & Sinclair, 1980). For the isolation studies, flower debris and bunch sections were incubated on Kerssies *B. cinerea* selective medium (Kerssies, 1990) or on paraquat-chloramphenicol agar. Preliminary studies showed that compounded included in the selective media had no effect on the fungal antagonists. The percentage flower debris and bunch sections yielding a fungal antagonist or the pathogen at a specific position were recorded after 14 days. *T. pullulans* is generally not detectable on grape berries 4 days after application (Williamson, 1997). Presence of the yeast was therefore not monitored.

Results

Antagonist development

Climatic conditions in the Chardonnay vineyard ranged from cool and wet during the first season to hot and dry in the last season. It was generally dry with no rainfall during the 24 h-period following the antagonist application, and prevailing temperatures were lower than the optimum temperatures needed for maximum development by the antagonists.

Stamen, calyptra and aborted embrios were colonised primarily by *T. harzianum*. The organism developed from 100% of the floral debris in the first season, but colonisation was poor (<30%) in the following seasons. *U. atrum* and *G. roseum* colonised floral debris at low levels (<40%) in the first season, but in the following two seasons these two antagonists did not develop from floral debris.

The different tests showed that *T. harzianum* occupied all the positions in the bunches, and that colonisation was best during pea size stage. Most important, the organism showed preference for colonising primarily the bases of the pedicels and berries. At pea size stage, nearly 100% of these positions were colonised by the organism. Colonisation was also good at bunch closure, but inconsistent at véraison and harvest. Occupation was furthermore at much higher levels during the first than the following two seasons. *U. atrum* developed from rachises, laterals and pedicels during bloom, but was not found at these positions after the pea size stage. This suggests that the saprophyte resided in moribund tissue in bunches during full bloom to the pre-pea size stage, and is not likely to be found in living tissue. *G. roseum*, on the other hand, displayed poor colonisation and developed erratically and at low levels at these positions in bunches.

In both seasons rainy events which might lead to humid conditions in the Dauphine vineyard on the day of antagonist application were not recorded at any growth stage during the 3-day period prior to antagonist application. No rain fell during the 24 h-period following the antagonist application and prevailing temperatures were lower than the optimum temperatures needed for maximum development by the antagonists. Virtually none of the floral debris recovered from bunches yielded the organisms in both seasons. Only *T. harzianum* occupied the various positions in bunches from pre-pea size to bunch closure. However, colonisation levels were generally low and never exceeded 50%.

***Development of* B. cinerea**

In the 1996/1997 season, climatic conditions favoured the natural development of *B. cinerea* from bloom to bunch closure. In the 1997/1998 season, conducive periods were recorded only during the early and late pea-size stage. In 1998/1999 and 1999/2000, climatic conditions favoured the natural development of *B. cinerea* only during bloom. The different tests showed that pathogen occurred sporadically in flower debris and in bunches of both cultivars. It was therefore not possible to comment on the efficacy of the various antagonists in the four seasons during which the trials were performed.

Discussion

The study showed that the three fungal antagonists could each contribute to the reduction of *B. cinerea* in grape bunches by occupying the different positions available to the pathogen. However, they were not equally effective in colonising their niches, and colonisation levels fluctuated drastically in a season, between seasons, and between the two vineyards. This could be ascribed to two constrains. Firstly, climatic conditions in vineyards in the Western Cape province may not favour the establishment of the isolates of the agents used. Secondly, the antagonists are not effectively dispersed to the inner bunches by conventional spray apparatus after bunch closure. Thus, due to these constrains, it would be difficult integrating different antagonistic micro-organisms exhibiting different modes of action in a disease control program. Although no conclusive remarks could be made on the ability of the antagonists to control the pathogen, it is of interest to note that, during pea-size stage in 1996, when high levels of *B. cinerea* were recorded, *T. harzianum* controlled these infections in the pedicel and berry bases more effectively than any other treatment. The organism also survived at these positions for at least seven to eight weeks. These finding suggests that, amongst the biocontrol agents tested, *T. harzianum* has the greatest potential to be used as a component in an integrated disease control program.

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P10c: a new biocontrol agent against fire blight

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Abstract: We have selected a strain of *Pantoea agglomerans* which proved effective in controlling fire blight in experiments conducted on immature pear fruits and on pear flowers in the laboratory and on apple flowers in experimental orchards. This strain was selected for its ability to colonise rapidly the stigmatic surfaces of apple and pear flowers, the niche needed for development of the disease. In addition, P10c also produces a compound which inhibits the growth of the fire blight pathogen. A derivative of P10c, which is resistant to all the bacteriophages isolated so far and which is also resistant to streptomycin has been selected. Honey bees have been used to deliver this beneficial bacteria onto apple, pears and nashi flowers. The percentage of flowers colonised by P10c and the level of colonisation obtained using honeybees, resulted in a reduction of the disease to levels similar to that obtained with streptomycin.

Keywords: *Erwinia amylovora*, *Erwinia herbicola*, *Pantoea agglomerans*, fire blight, Blossom Bless

Introduction

Fire blight is a bacterial disease of apple and pear for which only few methods of control are available (Vanneste, 2000). Chemical control is mostly limited to the use of antibiotics or heavy metals, two types of compounds which present several disadvantages (Psallidas & Tsiantos, 2000). Copper derivatives are phytotoxic and have been found to accumulate in the soil leading to further toxicity problems, and repeated use of streptomycin can lead to selection of strains of *Erwinia amylovora*, the fire blight pathogen, resistant to this antibiotic (Jones & Schnabel, 2000; Vanneste & Voyle, 2002). Biocontrol using some non-pathogenic epiphytic bacteria has shown some promise (Vanneste, 1996; Johnson & Stockwell, 2000). This report is about a new strain of *Pantoea agglomerans* (formerly *Erwinia herbicola*) isolated from blossoms collected in a pear orchard in New Zealand which consistently reduced fire blight incidence on immature pear fruit in the laboratory. This strain called P10c also reduced incidence of fire blight on apple and pear flowers in experimental orchards when sprayed before inoculation with *E. amylovora*.

Aptitude to establish on and colonise apple and pear flowers is an important characteristic for bacterial strains we want to use as biocontrol agent of fire blight. P10c was selected among other potential biocontrol agents for its ability to colonise rapidly apple and pear flowers. After artificial inoculation of apple flowers in the laboratory, P10c reached populations of 10^6 to 10^7 cfu per flower within 48 hrs. This represents the carrying capacity of the stigmatic surfaces, i.e. the maximum number of bacteria which can grow on the stigmatic surface of an apple flower due to the limiting amount of nutrients available and/or due to the limiting space available for growth. P10c was also selected for its ability to produce a compound which inhibits the growth of *E. amylovora*.

Ability of P10c to protect against fire blight was initially assessed on cores of immature pear fruits in the laboratory. In all cases, immature pear fruits treated with P10c were less affected by fire blight than those treated with $MgSO_4$ (Figure 1).

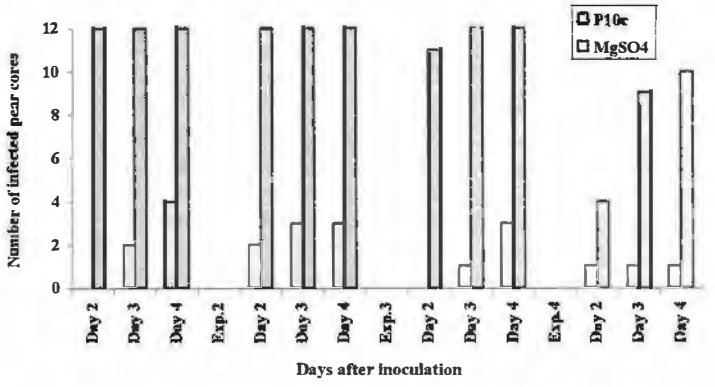

Figure 1. Number of fire blight infected pear cores that have been treated with P10c or $MgSO_4$ and inoculated with *E. amylovora*.

Ability of this strain of *P. agglomerans* to reduce incidence of fire blight on flowers was then demonstrated using pear branches in the laboratory. Branches of pear cultivar Conference were brought from the orchard and all open flowers were removed. The branches were kept in vases filled with water for the remaining flowers to open. These flowers were then treated with either water or P10c (2×10^8 cfu/ml) and inoculated two days later with *E. amylovora* strain 1540 at 3×10^6 cfu/ml. Presence of exudate on flowers, signalling fire blight infection, was assessed every day. Flowers treated with water only or with P10c only never showed signs of fire blight infection. Flowers treated with P10c showed fewer symptoms than those treated with water (Table 1).

Table 1. Percentage of pear flowers showing fire blight infection after treatment with P10c or water and inoculation with *E. amylovora*

Days after inoculation	Water	P10c
5	17	0
6	61	10
7	76	22
10	82	38

We then conducted a series of experiments in three different apple orchards located in the Waikato (centre of North Island of New Zealand). Bacterial suspension of P10c ($1\text{-}2 \times 10^8$ cfu/ml), streptomycin (100 ppm) or water was sprayed to run off on newly open blossom clusters. The next day the same blossom clusters were sprayed with an aqueous suspension of *E. amylovora* ranging from 5×10^5 to 1×10^7 cfu/ml depending on the experiment. The

number of healthy and diseased clusters was recorded several weeks after inoculation. In all these experiments the level of control obtained with P10c was similar to that obtained with streptomycin (Table 2). However, the short delay between treatment and inoculation favoured streptomycin (Vanneste1996) while it gave very little time for P10c to establish on the flowers.

Table 2. Percentage of apple clusters showing signs of fire blight infection

	Treatment		
Experiment	Water	Streptomycin	P10c
Ruakura[I]	53.0	5.2	9.1
University	46.0	4.0	6.0
Ruakura[II]	28.7	16.0	15.3
Blands	74.9	43.9	51.5

We selected a derivative of P10c which is resistant to all the bacteriophages we have isolated. We checked that this derivative was not impaired in its ability to colonise apple or pear flowers or in its ability to inhibit *E. amylovora* on plate. This derivative is also resistant to streptomycin due to a mutation in codon 43 of the *rpsL* gene. This allows using streptomycin and P10c together.

We previously demonstrated that honeybees could deliver *E. herbicola* onto apple and pear flowers (Vanneste, 1996; Vanneste et al., 1999). In this study we compared the level of control conferred by P10c delivered using honeybees to that of P10c and streptomycin sprayed directly onto pear flowers. Two-year-old potted pear trees were located on several commercial orchards, some of which had bee hives fitted with a pollen insert filled with a powder containing 10^9 cfu/g of P10c. This is 100 times less concentrated than the powder used previously (Vanneste et al., 1999). Four days after the start of the experiment, trees were removed from the orchards and brought into a glasshouse where some of them were sprayed with either streptomycin (100 ppm) or with an aqueous suspension of P10c before being inoculated the next day with *E. amylovora*. The percentage of healthy clusters from the trees treated with P10c (using either honeybees or a sprayer) were not significantly different from that of trees treated with streptomycin but were significantly different from that of trees that were not treated (Figure 2). This demonstrates that honeybees can be used to deliver antagonistic bacteria onto pear flowers to reduce incidence of fire blight. We also demonstrated the transfer of P10c, by honeybees or other pollinating insects, from flowers initially colonised to newly opened flowers. This transfer of beneficial bacteria lead to the protection from fire blight of flowers that opened after the initial treatment whether using a sprayer or honeybees.

P10c has proven to be one of the most effective strains of bacteria for control of fire blight. It is the major component of a commercial product called Blossom Bless now on sale in New Zealand. This powder can be sprayed directly onto flowers or delivered to flowers using honeybees. In both cases, under best conditions, 100% of the flowers randomly sampled in treated commercial orchards were colonised by P10c.

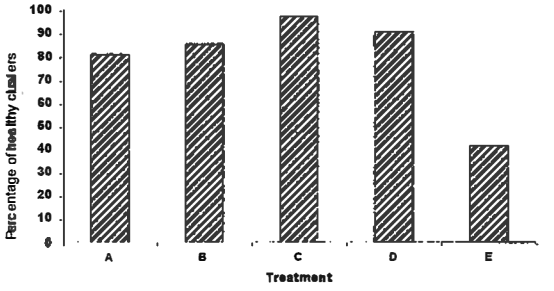

Figure 2. Mean percentage of healthy clusters after inoculation with *E. amylovora*.
A: Trees from orchards in which P10c was distributed by honeybees; B and C: Trees sprayed with a suspension of P10c $2.5x10^7$ and $5x10^8$ cfu/ml respectively; D: Trees sprayed with streptomycin 100 ppm; E: Trees not treated.

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Biocontrol of *Sclerotinia sclerotiorum* by *Trichoderma* spp. resistance-inducing isolates as modified by spatial, temporal and host plant factors

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Abstract: Biocontrol of white mold (*Sclerotinia sclerotiorum*) by *Trichoderma* spp. isolates was tested on cucumber, lettuce, bean and tomato plants. Leaves were infected by means of mycelium discs placed on their adaxial (upper) side, whereas the *Trichoderma* was applied as a suspension, either to the root zone or to the leaves. Plants were maintained in a greenhouse under conditions of high humidity at 20-25°C. Disease control was achieved on the various hosts, but the level of control was affected by factors such as the *Trichoderma* isolate tested, the host species, the plant organ treated, the pathogen isolate, the nature of the biocontrol preparation and the time interval between biocontrol application and pathogen inoculation. An interaction between different factors was found throughout the experiments. Seven isolates of *Trichoderma*, including isolate T39 (of the commercial product TRICHODEX) were tested. Increased concentration of T39 conidia (from 1 to 5×10^5/ml) and application to the leaves and to the roots, as a formulation instead of as naked conidia, improved disease suppression on cucumber, tomato and lettuce. This happened in some of the isolates, probably because of their differing requirements. Application of the biocontrol agents 1-5 days before inoculation resulted in better disease suppression by certain isolates. Application to lower leaves resulted in disease control similar to that by soil application in cucumber and bean. Treatment of the abaxial side of the leaves of cucumber and bean resulted in suppression of the adaxial infection also. Dead cells of T39 suppressed white mold as effectively as live cells. The results demonstrate variability in efficacy of biocontrol of *S. sclerotiorum*, among the *Trichoderma* isolates and treated host species Nevertheless, disease reduction of above 70% could be achieved. Apparently the *Trichoderma* activity can be at least partly related to induced resistance. Given that there were spatial and temporal differences between *Trichoderma* application and pathogen inoculation, it can be concluded that the signal passes from roots or lower leaves to upper leaves and through the leaf tissue. A similar effect can be related to dead cells of *Trichoderma*.

Key words: induced resistance, *Trichoderma harzianum*

Introduction

Sclerotinia sclerotiorum is a necrotrophic pathogen that affects many plant hosts. It causes white mold on various plant parts including stems and stem bases, leaves, flowers and fruits, and it may result in severe yield losses. The pathogen produces sclerotia that may survive in unfavorable conditions for long periods. Apothecia that are formed on the sclerotia produce acospores that can spread easily through the air. Studies of biocontrol management of the disease have examined the use of parasites of the sclerotia, such as *Coniotirium minitans,* in the soil, and the use of attenuated isolates of the same species (hypovirulence) (Elad & Freeman, 2002). Recently it was reported that sprays of *Trichoderma harzianum* T39 (TRICHODEX) controlled the disease in commercial greenhouses (Elad, 2000).

The general objective of the present research was to characterize control of *S. sclerotiorum* by *Trichoderma* isolates. Specifically, we examined: i. whether control was achieved when *Trichoderma* and *Sclerotinia* were applied to separate sites; ii. whether time separation would affect the control efficacy; iii whether formulation of the *Trichoderma* conidia would promote biocontrol activity; iv. whether viability of the *Trichoderma* cells was important for biocontrol activity; v. the importance of the *Trichoderma* isolate; and vi. the effect of the host plant.

Materials and methods

Biocontrol of white mold (*S. sclerotiorum*) by *Trichoderma* spp. isolates was tested on cucumber, lettuce, bean and tomato plants. Leaves of the plants were infected by means of mycelium discs (from the edges of 4-day-old PDA cultures) placed on their adaxial (upper) side. Seven isolates of *Trichoderma*, including isolate T39 (of the commercial product TRICHODEX) were tested. The formulation of the isolates was similar to that of TRICHODEX (Elad, 2000). The biocontrol agents were applied as a spore suspension. When a formulation was compared with naked conidia, care was taken to apply the same cell concentration. Dead cells of the *Trichoderma* isolates were obtained by heating to 100°C for 20 min, and the cells were washed by centrifugation. Some of the treatments involved spatial separation of the sites of *Sclerotinia* inoculation and of *Trichoderma* application. In all cases the inoculation was performed on the upper leaves. *Trichoderma* was applied to one of the following sites: i. whole canopy; ii. underside of the infected leaf; iii. lower leaf; vi. roots. In one set of experiments the time of *Trichoderma* application was separated from that of the pathogen inoculation. Plants were maintained in a greenhouse at 20-25°C, and were covered with polyethylene bag to maintain high humidity. Experiments were usually constructed to test one to three of the parameters mentioned above. In all experiments, disease severity (lesion area) was assessed periodically and the data was used to calculate the area under disease progress curves (AUDPC) for each treatment.

Results and discussion

Seven isolates of *Trichoderma*, including isolate T39 (of the commercial product TRICHODEX) were tested. The activity of the *Trichoderma* isolates varied with the host plant (results not shown). Control of the disease was achieved both when the *Trichoderma* isolates were applied to the same site as the pathogen and when they were separated from it. For instance, on tomato, T39 reduced tomato white mold by 68 or 72% when applied to the roots or sprayed on the leaves, respectively, whereas the rest of the isolates were significantly less effective. Increased concentration of T39 conidia (from 1 to 5×10^5/ml) improved control efficacy (results not shown).

Application of the biocontrol agents 1-5 days before inoculation resulted in better disease suppression by certain isolates. However, this was not evident on cucumber (Table 1). Application to lower leaves of cucumber and bean resulted in similar disease control to that achieved by soil application (Table 1), the effect being more pronounced when plants were treated 3-5 days prior to infection rather than 0-1 days before infection. Treatment of the abaxial (under) side of cucumber and bean leaves resulted in suppression of the adaxial infection also (Table 2).

Formulation of the *Trichoderma* isolates resulted in better control of white mold on cucumber and lettuce than the use of conidia alone, as shown for T39 treatment (Table 3). However, the effect was observed with only some of the isolates, probably because their

requirements differed. Dead cells of T39 suppressed white mold as effectively as live cells (Table 3). Use as a formulation improved disease suppression on tomato, too, when applied to the leaves and also, to a lesser extent, when it was applied to the roots (results not shown).

Table 1. Control of *Sclerotinia sclerotiorum* white mold on cucumber and bean leaves by *Trichoderma* isolates. Effects of time of application and site of application

Trichoderma application	Time separation between *Trichoderma* application and *Sclerotinia* inoculation		
	0	3	0
Experiment	1 AUDPC		2 AUDPC
Control	845 a	974 a	2502 a
Canopy	202 b	399 b	
Soil	363 b	392 b	
Lower leaf			520 b
Soil			420 b
Crop	Cucumber	Cucumber	Bean
Isolate	T161	T161	T39

Numbers represent area under the white mold rot progress curve (AUDPC).
Numbers in each experiment followed by a common letter are not significantly different ($P \leq 0.05$).

Table 2. Control of *Sclerotinia sclerotiorum* white mold on bean leaves by *Trichoderma* isolates applied to the leaf underside

Formulation	Site of application	Bean	Cucumber
Control		313 a	836 a
T116	Leaf underside	199 b	606 b
T39 (TRICHODEX)	Leaf underside	45 c	263 c

Numbers represent area under the white mold rot progress curve (AUDPC).
Numbers in each column followed by a common letter are not significantly different ($P \leq 0.05$).

In general, control of the disease was achieved on the various hosts, but the level of control was affected by factors such as the *Trichoderma* isolate tested, the host plant species, the plant organ treated, the pathogen isolate, the nature of the biocontrol preparation and the time interval between biocontrol application and pathogen inoculation. Interactions among various factors were found throughout the experiments. Highlights of the results are presented in this report. The results demonstrate variability in biocontrol of *S. sclerotiorum*, both among the *Trichoderma* isolates and among the treated host species, but disease reduction by over 70% could be achieved. Apparently the *Trichoderma* activity can be at least partly related to induced resistance. Given that there were spatial and temporal differences between *Trichoderma* applications and pathogen inoculation, it can be concluded that the signal passes from roots or lower leaves to upper leaves and through the leaf tissue. A similar effect can be related to the dead cells of *Trichoderma*. Formulation and increased concentration of the biocontrol fungus may improve its activity.

Table 3. Control of *Sclerotinia sclerotiorum* white mold on lettuce and cucumber leaves by *Trichoderma harzianum*. Effects of site of application, formulation of the *Trichoderma* and of biocontrol agent viability

Treatment		Conidia	Formulation	Conidia	Formulation
		Experiment 4 AUDPC		Experiment 5 AUDPC	
Control	Live	1396 a	-	1500 a	-
Canopy spray	Live	410 b	169 c		
Soil application	Live	502 b	181 c		
Canopy spray	Live			500 b	110 c
Canopy spray	Dead			700 b	200 c
Crop		Lettuce		Cucumber	
Isolate		T39		T39	

Numbers represent area under the white mold rot progress curve (AUDPC).
Numbers in each experiment followed by a common letter are not significantly different ($P \leq 0.05$).

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Effect of environmental factors on growth, pycnidial production and spore germination of four apple scab biocontrol agents and influence of pseudothecia maturity on biological activity

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Abstract: The potential as biocontrol agents of the apple scab pathogen, *Venturia inaequalis* and the effect of environmental factors on growth, pycnidial production and spore germination of four isolates of *Microsphaeropsis* were examined on cultnre media and leaf discs. *V. inaequalis* ascospore production was reduced by 52.0, 77.4, 88.9, and 95.2% on leaf discs treated with the isolates IMI294735, P176A, DAOM198536, and P130A, respectively. For all isolates, optimum temperature for mycelial growth was 25°C. More pycnidia were produced on culture media than on leaf discs. On culture medium, optimum temperature was from 15 to 25°C, while on leaf discs it varied among isolates. Pycnidial production was inhibited by darkness and a minimum of 8 h of light d^{-1} was required. Spores of isolates P130A and DAOM198536 germinated at temperatures above 15°C and at pH between 4 and 5, as compared to 10°C and pH of 4 to 8 for isolates P176A and IMI294735. The best candidates were P130A identified as *M. ochracea* and DAOM198536 and optimum conditions for growth, pycnidial production and spore germination were temperatures between 20 to 25°C, pH around 4 and light.

The strategy of biocontrol proposed consists in applying *M. ochracea* in the autumn on fallen leaves. It was thus important to evaluate the effect of *V. inaequalis* pseudothecia maturity on the antagonistic activity in order to better define the window of application. Sterile leaf disks were inoculated with a mixture of *V. inaequalis* isolates and spores of *M. ochracea* (4.5 X 10^5 conidia per mL) applied 0, 2, 4, 6, 8, 10, 12, 14, 16 days later. After an incubation period of 3 weeks at 18C, 4 weeks at 4°C, 12 weeks at 10°C and 3 weeks at 20°C, the number of ascospores per cm^2 of leaf disk was counted. A similar experiment was conducted under field conditions where *M. ochracea* was applied on scabbed leaves at 2-week intervals from August to November. Leaves were over wintered on the orchard floor and ascospores production evaluated weekly from April to June. For both experiments, ascospores inhibition ranged from 100 to 95% when *M. ochracea* was applied before pseudothecial initiation and dropped to about 70% when applied later. Under orchard conditions, best ascospore inhibition was observed when the biocontrol agent was applied in September or late April.

Key words: apple scab, *Microsphaeropsis* spp., *Venturia inaequalis*

Introduction

Apple scab (*V. inaequalis* (Cke.) Wint.), is one of the most important diseases of apple. Since the dawning of organic fungicides in the 1940's, fungicides became the sole means for managing this disease. Over the years, there have been several attempts to develop biocontrol strategies, but until recently, none had reached the point where it could be used commercially (Carisse & Dewbney, 2002). *V. inaequalis* overwinters as pseuodothecia formed in apple leaf litter. Ascospores produced in the spring are the main source of inoculum. The most promising approach to apple scab biocontrol involves in applying a biocontrol agent in autumn, which will interrupt overwintering of the perfect stage (Carisse & Dewdney, 2002).

In 1996, Bernier et al. isolated 189 fungi from dead apple leaves. Some of these fungal isolates were evaluated for their ability to inhibit ascospores production (Philion et al., 1997). Two *Microsphaeropsis* isolates (P130A and P176A) were selected and further tested in the field (Carisse et al., 2000). For commercial uses, proper timing of the antagonist is crucial. Treatment in the fall should be made to favor leaf colonization. If the antagonist is applied on the tree canopy before leaf fall, the environment at the leaf level may not favor leaf colonization by the antagonist or allow it to maintain its population at a high level. On the other hand, if the antagonist is applied on the ground after leaf fall, weather conditions in northern regions may be unfavorable to the antagonist activity. Before deciding which spray strategy is most promising, it is important to determine the effect of the environment on leaf colonization and the importance of pseudothecia development. Therefore, the objectives of this study were to obtain quantitative information on the effect of temperature, pH and light on fungal growth, pycnidial production and spore germination of *M. ochracea* and three related species and to evaluate the effect of pseudothecia development on the inhibition of ascospore production.

Materials and methods

Microsphaeropsis ochracea P130A, and *Microsphaeropsis* sp. P176A were isolated from dead apple leaves collected in Quebec orchards (Bernier et al., 1996; Carisse & Bernier, 2002), *Microsphaeropsis* sp. DAOM 198536 was isolated from apple fruits and was obtained from the Canadian Fungal Culture Collection and *Microsphaeropsis arundinis* IMI 294735 was isolated from apple leaves was obtained from the International Mycological Institute.

Effect of environmental factors

To test the effect of temperature on mycelial growth, a mycelial plug of 6 mm in diam. was placed in the center of a PDA dish. The dishes were incubated at different temperatures and radial growth was measured after 1 and 2 weeks of incubation. To study pycnidial production on culture media, 50 µl of spore suspension was inoculated on PDA dishes and spread onto the entire surface of each dish. To test the effect of temperature, dishes were incubated for 3 days at room temperature to allow hyphal growth prior to transfer to growth chambers adjusted to 5, 10, 15, 20 and 25°C, with a photoperiod of 12 h of light per day. To test the effect of pH, PDA medium was buffered prior to autoclaving with a citrate-phosphate solution to obtain pH values of 4.0, 5.0, 6.0 and 7.0 and with a phosphate buffer solution to obtain a pH of 8.0. The inoculated dishes were incubated in growth chambers adjusted to 20°C, with a photoperiod of 12 h of light. To test the effect of light regime, the inoculated dishes were incubated at 20°C under daylight of 0, 8, 12, 16, and 24 h, provided by fluorescent and incandescent lights. The same protocol was used to study the effect of temperature and light regime on spore germination on culture media with the following modifications: the spore suspensions were spread onto water agar and the dishes were transferred into the growth chambers immediately after inoculation. Pycnidial production was evaluated on two samples of 3 mm in diameter. Samples were collected every two or three days over a period of 14 to 18 days, depending on experiments. Spore germination was assessed after 16, 24 and 40 h of incubation. A total of one hundred spores from each dish were examined. A conidium was considered germinated when the germ tube was at least the length of the spore.

The effects of temperature and light on pycnidial production were also examined on apple leaf discs sterilized by irradiation. The spore suspensions were prepared as previously described and 100 µl of suspension was inoculated on each leaf disc. The effect of temperature and light regime was tested as described above. The density of pycnidia on each leaf disc was evaluated by counting the number of pycnidia in two binocular fields, which

corresponded to 25.5% of the surface of the disc. Observations were made every two or three days over a period of 13 to 15 days, depending on the experiment.

Pseudothecia maturity

Sterilized apple leaf discs were inoculated with a mixture of 7 isolates of *V. inaequalis*. Sequential inoculations of the leaf discs with *M. ochracea* were done 0, 2, 4, 6, 8, 10, 12, 14, and 16 days later. The discs were incubated as described by Philion et al. (1996). The ascospore production was evaluated at the end of the incubation period (19-21 weeks), which was determined, based on ascospore maturity on leaf discs inoculated with *V. inaequalis* only. At two-week interval, from mid-August to the end of October and during the following May, scabbed apple leaves, from McIntosh trees, were inoculated with a spore suspension (9 X 105 spores/mL.) of *M. ochracea*. Immediately after inoculation, the sprayed leaves were enclosed in a mesh bag. For each inoculation, 10 branches were inoculated per treatment (*M. ochracea* and control). During the first week of November, all bags were removed from the trees and fixed to the ground to over winter. The following spring, before the beginning of the ascospore ejection period, the leaves were placed in wooden traps and the amount of ascospores ejected was evaluated weekly.

Results

For all isolates, mycelial growth increased with increasing temperature following a similar pattern. Temperature had a significant effect on pycnidial production on culture media. Only few pycnidia were produced at 5°C. At 10°C, isolates DAOM198536 and IMI294735 produced significantly more pycnidia than the other isolates. At temperatures of 15, 20 and 25°C, there were no significant differences between the numbers of pycnidia produced by the 4 isolates. Overall, pH had a significant effect on pycnidial production per cm^2 ($P \leq 0.0001$) but large variations were observed among observations. For all isolates, optimum pH for pycnidial production was 4 and for most isolates only a few pycnidia were produced at pH above 4. Less pycnidia were produced under complete darkness as compared to 8, 12, 16 and 24 h of light per day.

Spore germination began after 16 h at 10°C for isolate IMI294735 and at 15°C for the other isolates. Spores of isolates P176A and IMI294735 germinated significantly faster than spores of the other two isolates. Almost all spores of isolates P176A (93.2%) and IMI294735 (90.4%) germinated after 16 h at 20°C, as compared to only 22.2 and 23.9% for isolates P130A and DAOM198536. Similar effects of temperature on spore germination were observed after 24 h, with a delay in germination for isolates P130A and DAOM198536.

After 24 h of incubation, spore germination of isolates P130A and DAOM198536 was almost completely inhibited at pH above 6. Optimum germination was observed at pH 5, with percent spore germinations of 72.5 and 90.7%, respectively. For the isolates P176A and IMI294735, percent spore germination was optimum at pH of 4 to 6, with averages of 99.3 and 99.5%. Spore germination then decreased to 58.6 and 58.4%, respectively, with increasing pH from 6 to 8.

Less pycnidia per cm^2 was produced on leaf discs than on culture medium and greater differences among the isolates were observed on leaf discs. At 5°C, none of the isolates produced pycnidia on apple leaf discs. At 10°C, isolates P176A and IMI294735 produced significantly more pycnidia than the other two isolates. Optimum temperature for pycnidial production was 25°C for isolates P130A and IMI294735, 20°C for isolate DAOM198536, and 15 to 20°C for isolate P176A. Few pycnidia were produced under complete darkness. For most light regimes, isolate P176A produced more pycnidia than the other isolates and pycnidial production increased with increasing light duration. For the other isolates, similar

amounts of pycnidia were produced under 8, 12, 16, and 24 h of light per day. Under controlled conditions, more than 90% of the ascospore production was inhibited when *M. ochracea* was applied within 4 weeks after the inoculation with V. inaequalis. When the interval between inoculation with *V. inaequalis* and *M. ochracea* increased from 6 to 16 weeks the ascospore inhibition dropped to 60 to 74%. Under orchard conditions, best ascospores inhibition was observed when *M. ochracea* was applied early in the fall (before September 8) or in early spring (May 4).

Discussion

From the results of this study, it is evident that pycnidial production and spore germination are optimized at a temperature between 20 and 25°C and a pH between 4 and 5. Light is required for both pycnidial production and spore germination. However, optimum conditions for growth, reproduction and germination of the biocontrol agent are not necessarily those for best biocontrol activity. Isolates P130A and DAOM198536 are the most promising candidates for biocontrol of the perfect stage of *V. inaequalis*. For mass production of inoculum, temperature should not be a problem, as pycnidia were produced on culture media at temperatures between 15 and 25°C. If spores are to be used as inoculum, light may be a problem for large containers, as a minimum of 8 h of light per day was required for pycnidial production. It becomes apparent that orchard application of these biocontrol agents should be made when the temperature is above 15°C for at least 24 h and preferably on senescent leaves (lower pH). Pseudothecia maturity should not be a limiting factor. Spring treatments under optimum temperature for spore germination and mycelial growth should also be considered in areas where it is possible. The information acquired in this study will serve as a basis for the development of these biocontrol agents. However, information on the relationship between *Microsphaeropsis* sp. and apple leaves and between *Microsphaeropsis* sp. and *V. inaequalis* will be necessarily to optimize timing of application.

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Biocontrol on harvested fruit

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Integrated control of postharvest decay using yeast antagonists, hot water and natural materials

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Abstract: The past decade has seen a steady increase in the interest of finding alternatives to the use of synthetic fungicides for postharvest disease control. In particular, this has led to considerable research on the use of microbial antagonists as protective agents in much the same way as packing houses use synthetic fungicides for disease control. Biological products such as Aspire, BioSave, and Yield Plus, based on either yeast or bacteria, are now available in the marketplace and several new products are in various stages of development. The success of these products, however, remains limited. This is for several reasons, among which is the variability experienced in the efficacy of these products, and the lack of understanding how to adapt "biological approaches" to a commercial setting. In recent years we have been developing an integrative approach in which various physical and biological treatments are implied. This approach relies on first application of hot water treatment that disinfects and eradicates existing infections followed by microbial antagonists that protect the commodity from future infections. Combining antagonists with natural materials such chitosan, lytic enzymes as well as food-grade preservatives (e.g. sodium bicarbonate, potassium sorbate, and calcium chloride) has been also evaluated. Collectively the results clearly show that performance of yeast biocontrol agents could be augment by development of a combination of complementary biological approaches for additive and/or synergistic effects. Combining yeast antagonists with either hot water pretreatment or mixing with antimicrobial substances shown to consistently offer an economically sufficient level of disease control that will warrant their acceptance as a viable alternative to synthetic fungicides.

Key words: heat treatment, salt solutions, induced resistance, yeast, bacteria, chitosan

Introduction

Due to public demand, there is strong pressure to reduce or eliminate the use of synthetic chemicals for control of postharvest diseases of fruits and vegetables (Wilson et al., 1996). This demand is supported by increased concerns about the health risks associated with the use of these chemicals, especially in relation to consumption by children. Problems associated with pathogen resistance and the economics of re-registration of these chemicals further makes the search for effective alternatives an attractive line of research. In the present report, an overview of various alternative approaches to postharvest disease control is provided. In particular, attention is given to the integration of various alternatives with the use of biocontrol agents. Since this topic is quite broad, the overview does not intend to be all inclusive but rather draw attention to a number of alternatives that have recently been demonstrated to show good potential.

Control of postharvest decay

Microbial antagonists

Considerable research effort has been devoted to identifying microbial antagonists that effectively control postharvest diseases of fruit, vegetables, and grains (Wilson et al., 1996; Janisciewicz, 1998; Wilson et al., 1994). At least two, yeast-based, products, are now commercially available (Aspire based on *Candida oleophila*, and Yield Plus based on *Cryptococcus albidus*), and several more are in the advanced stages of the commercialization process (El Ghaouth et al., 2000). The products Biosave-100 and Biosave-110, based on the bacterium, *Pseudomonas syringae*, are also available for postharvest disease control (Janisiewicz et al., 1997). Although antagonistic yeasts have been shown to protect a variety of fruit, their efficacy under semi-commercial conditions is often lower than chemical control (Droby et al., 1998; El Ghaouth et al., 2000). In large-scale tests the use of antagonists often needs to be supplemented with low doses of synthetic fungicides in order to provide a level of disease control equivalent to synthetic fungicides (Droby et al., 1998). The use of microbials is especially useful as part of a resistance management program where they have been shown to control resistant strains of pathogens not affected by the prevailing synthetic fungicide. A principal goal in the development of "second generation" products is improving the ability of yeast antagonists to successfully control postharvest diseases under a wider range of conditions and with less variability. Additionally, the ability to control pre-existing infections, as is possible with synthetic fungicides, is also highly desired (El Ghaouth et al., 2000). For this reason, the use of additives, or physical treatments (heat, UV-light), in combination with the antagonists have been explored.

Heat treatment

The exposure of various fruits to high temperatures (50–60°C) either as a wet or dry treatment has been demonstrated to have a significant impact on controlling both postharvest diseases and fruit quality. A short hot water brushing treatment (20 s at 62°C) controlled green mold (*Penicillium digitatum*) decay in organic citrus fruit (Porat et al., 2000) and improved color retention in litchi fruits (Lichter et al., 2000). Thermal postharvest treatments have also improved the quality and shelf life of pomegranate (Artes et al., 2000). Prestorage hot air treatment of apples at 38°C, in combination with calcium infiltration, has also been shown to significantly reduce storage decay caused by *Penicillium expansum* and *Botrytis cinerea* (Klein et al., 1997) and has also been used with the application of a biocontrol agent, *Pseudomonas syringae* (Conway et al., 1999). Significant control of postharvest diseases of stone fruit has also been observed when heat treatments are combined with the use of calcium chloride or antagonistic yeast (Wisniewski, unpublished data). Presumably, the effect of the heat treatment is due to direct effects on the pathogen and also the induction of resistance in the fruit. While it does not appear that high temperature treatments alone will control postharvest diseases, it is becoming apparent that the use of heat in combination with other approaches may lead to an integrated approach that would be commercially acceptable.

Salt solutions

Both sodium and potassium bicarbonate salts have long been known to have fungicidal activity against foliar pathogens (Corral et al., 1988; Kuepper et al., 2000). Evidence also indicates that various salt solutions can be used in a postharvest environment to control decay. Infiltration of calcium chloride into apples has also been shown to control postharvest disease, delay senescence, and reduce physiological disorders (Conway, 1982). Smilanick et al. (1997, 1999) demonstrated that green mold (*Penicillium digitatum*) of citrus could be effectively controlled with solutions of sodium carbonate and bicarbonate. Heating the solutions to 50°C improved the efficacy of the salt solutions. The salt solutions were also compatible with the

use of imazalil and biocontrol agents. Droby et al. (1997) and Wisniewski et al. (1995) demonstrated that both calcium and magnesium ions can be toxic to postharvest decay fungi and that salt solutions containing these elements can be used to enhance biocontrol activity of the yeast, *Candida oleophila*, the antagonist used in the commercial product, Aspire. More recently, Wisniewski et al. (1998) have demonstrated the activity of a wide range of "soft" or generally-regarded-as-safe (GRAS) chemicals against postharvest decay fungi. The compatibility and use of these compounds with biocontrol agents is being evaluated.

Bioactive compounds with microbial antagonists

Many of the alternative approaches discussed in this review do not control decay under commercial conditions to the same level as that of the currently-used synthetic fungicides. This has mostly been attributed to high inoculum pressure. When two or more of the alternatives are used together, however, the potential for obtaining acceptable levels of decay control, under a wider array of conditions, is much greater. Such an integrated approach has been pursued in combining a yeast antagonist (*Candida saitoana*) with various bioactive compounds (El Ghaouth et al., 2000). In this approach the antagonist has been used in combination with either glycochitosan or the sugar 2-deoxy-D-glucose. Another approach has been combining the yeast with low concentrations of lysozyme (El Ghaouth, unpublished data). The addition of these compounds has been shown to significantly improve the performance of the yeast and provide better decay control than the compound alone. The improvement of biocontrol agents is believed to be brought about by the direct action of the additive on the pathogen and also by the elicitation of defense mechanisms in the host. It is expected that such integrated approaches will lead to the development of products that can be used reliably in a commercial setting.

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Pre- and post-harvest practical application of *Pichia anomala* strain K, β-1,3-glucans and calcium chloride on apples: Two years of monitoring and efficacy against post-harvest diseases

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Abstract: Biological treatment based on a powder of *Pichia anomala* strain K (10^7 cfu/ml), β-1,3-glucans (YGT 2 g/l) and $CaCl_2.2H_20$ (20 g/l) was pre- or post-harvest applied against *Botrytis cinerea* and *Penicillium expansum* on apples under practical conditions. During the first year, the two highest protective levels were obtained with the pre-harvest standard chemical program consisting in 4 successive fungicidal treatments (99.6 %) and with the pre-harvest high volume spraying of the triple composition applied 12 days before harvest (82.6%). During the second year, post-harvest Sumico treatment and post-harvest biological treatment, both by dipping the apples, offered the highest protective levels (84.9 and 69.0 % respectively). A density threshold of 10^4 cfu of strain K/cm^2 of apple surface seemed to be required just after harvest in order to obtain a high protective activity whatever the mode and the time of application. Variations of meteorological conditions between both years were in accordance with strain K population density and efficacy differences observed in case of pre-harvest biological treatments.

Key words: Biological control, *Pichia anomala*, *Botrytis cinerea*, *Penicillium expansum*, apples, practical application, formulation

Introduction

Pichia anomala strain K was previously selected for its high antagonistic activity (even after its mass production and drying) on Golden Delicious apples against *Botrytis cinerea* and *Penicillium expansum*, two wound pathogens provoking economically important losses on storage rooms (Jijakli et al., 1999). Nevertheless, the use of a single antagonist has been sometimes criticized for not providing a reliable protective activity when used under commercial conditions. A proper formulation should be able to stabilize this activity by increasing the survival and/or the antagonistic properties of the biocontrol agent. YGT (containing 71 % of glucans) applied in combination with *P. anomala* strain K offered a higher and longer efficacy than this antagonistic strain used alone (Dicburt et al., 2001). Furthermore, triple composition based on *P. anomala* strain K (10^5 cfu/ml), β-,3-glucans (YGT, 2 g/l) and $CaCl_2.2H_20$ (20 g/l) lead to a higher protective percentage (up to 100%) than the percentage obtained by the sole strain K (10^7 cfu/ml) against both pathogens on apples under controlled conditions.

In this context, our objectives consisted in (1) evaluating the efficacy of the composition including strain K in practical conditions during two years and (2) assessing the population densities of the biocontrol agent in relation to its mode of application and meteorological conditions.

Material and methods

Apple treatments

Apple treatments were carried out in collaboration with the Royal station of fruit research of Gorsem (Belgium). During two successive years (2000 and 2001), the experimental Golden Delicious orchard (planted in 1995) was located in Melveren (Belgium). Temperature, rain and R.H. were determined with a meteorological station located in the orchard. Temperature mean (°C) and total rain (l/m^2) were calculated for each day. Golden Delicious apples were treated with a powder of strain K (10^7 CFU/ml) produced by CWBI (Université de Liège, Belgium) and supplemented with β-1,3-glucans (YGT, 2 g/l) and $CaCl_2.2H_20$ (20 g/l). This triple composition was applied 12, 5 or 2 days before harvest during the first year (15, 7 or 3 days during the second year) by spraying at low (300 l/ha) or high (1000 l/ha) volume. The different batches were artificially wounded just after harvest (4 wounds/apple). The triple composition including strain K was applied on untreated apples (with no chemical nor biological treatments) by dipping or drenching one day after harvesting. The following day, all the batches were artificially inoculated with *B. cinerea* (10^6 spores/ml) and *P. expansum* (10^5 spores/ml). Three controls were carried out as follows: 1) a standard program based on pre-harvest chemical treatments by spraying Bavistin, Phytocap, Sumico and Euparen, respectively 4, 3, 2 and 1 week before harvest, 2) a post-harvest standard treatment with Sumico (1 g/l) by apple dipping, 3) an artificial inoculation of pathogens on untreated apples. Four repetitions of 12.5 kg of fruits were used per treatment. Apples were successively stored at 1°C (15 days), 15°C (1 month) and 20°C (15 days) during the first year of trial (or two days at 20°C, 15 days at 1°C and 65 days at 20°C during the second year) before assessing the infection severity. During the first year, an index of severity (IS) was calculated (IS = number of class 1 lesions + 2 x number of class 2 lesions + n x number of class n lesions /total number of fruits) while lesion diameters were directly measured during the second year of trial. IS and lesions diameters were subjected to analysis of variance (SYSTAT) after log (first year) or square root (second year) transformation. The Duncan test was applied to compare the means at P = 0.001. A percentage of protection was also calculated based on the comparison of infection severity of treated batches and untreated but inoculated batch.

P. anomala *strain K monitoring*

The different treatments were applied on apples as above described but fruits were not wounded nor inoculated with pathogens and kept at 1°C during one month. After increasing periods, 8 apples per treatment were shaken separately during 20 minutes in 250 ml of KPBT 'washing' buffer (KH_2PO_4 0,034M, K_2HPO_4 0,016M, 0,05% tween 80, pH 6,5). The washing suspensions of the 8 fruits were mixed, serially diluted and plated on a semi-selective medium consisting in PDA supplied with 12.5 mg/l hygromycin B, 0.25 mg/l TMTD (containing thirame as active ingredient) and 5 mg/l Sumico (including 1.25 mg/l carbendazim and 1.25 mg/l diethofencarb). Petri dishes were incubated at 25°C during 4 to 5 days and yeast colonies of white colour were counted.

Results and discussion

During the first year, the best protection (99.6 %) against *B. cinerea* and *P. expansum* was obtained with the pre-harvest chemical treatments consisting in four successive applications of different fungicides. The highest percentages of protection (ranging from 40.3 to 82.6) by using biological treatment were observed in case of pre-harvest treatments with strain K (including YGT and $CaCl_2.2H_20$) whatever the application time. In opposite, the protective level of post-harvest apples treated with this triple composition was not significantly different

to the level observed for untreated apples. The best protective level (82.6 %) against *B. cinerea* and *P. expansum* was offered by the high volume spraying of strain K (12 days before harvest). This protective level was slightly higher (but not significantly) than the one recorded in case of post-harvest standard chemical treatment (74.7 %). More than 10^4 cfu of white yeasts/cm^2 of apple surface were observed just before pathogen inoculation in case of strain K pre-harvest high volume spray, 12 ($1.7\ 10^4$ cfu/cm^2) or 2 days ($3.1\ 10^4$ cfu/cm^2) before harvest (Figure 1A). This density level was never reached just before pathogen inoculation when apples were treated by dipping the post-harvest apples.

Suspecting a lack of stability of strain K suspension for post-harvest treatments, this suspension was mixed after each treatment during the second year of trial. A percentage of protection of 69.0 or 67.9 % against both pathogens was obtained with the post-harvest application of strain K respectively by dipping or drenching during this second year. These protective levels were lower (but not significantly) than the level reached with the post-harvest standard chemical treatment (84.9). In opposite, the highest percentages of protection obtained for pre-harvest chemical or biological (high volume spray 3 days before harvest) treatments were 59.4 and 38.9 % respectively. A density level of $1.6\ 10^4$ cfu of white yeasts/cm^2 of apple surface was recorded just before pathogen inoculation with post-harvest dipping of apples (Figure 1B) while lower density levels were observed at the same period for pre-harvest treatments (high volume spray 12 or 2 days before harvest).

Table 1: Efficacy of biological and chemical treatments in relation with their mode and time of application

	Treatments	First year		Second year	
Pre-harvest treatments	Low volume spray (12[a] or 15[b] days)	71.8[e]	bcdef[f]	26.9[e]	efghi[f]
	High volume spray (12 or 15 days)	82.6	b	36.8	efgh
	Low volume spray (5 or 7 days)	58.4	cdefgh	10.5	fghi
	High volume spray (5 or 7 days)	40.3	cdefgh	-11.0	hi
	Low volume spray (2 or 3 days)	56.3	cdefgh	-11.5	hi
	High volume spray (2 or 3 days)	65.7	cdefgh	38.9	defgh
	Chemical standard treatment[c]	99.6	a	59.4	bcdef
Post-harvest treatments	Dipping	-4.0	fgh	69.0	bcd
	Drenching	-8.1	fgh	67.9	bcde
	Chemical standard treatment[d]	74.7	bcde	84.9	ab
Control	Untreated apples	0.0	h	0.0	ghi

a and b = moment of treatment application before harvest during the first ([a]) or the second year ([b]), c = spraying of Bavistin, Phytocap, Sumico and Euparen at the authorised Belgian dose, respectively 4, 3, 2 and 1 week before harvest, d = dipping in Sumico (1 g/l), e = Percentage of protection based on the infection severity of untreated but inoculated batch, f = Duncan test was carried out on infection severity means (not shown). Treatments with a common letter do not differ significantly ($P \leq 0.001$).

The contradictory results of efficacy between years 2000 and 2001 for biological treatments could be partially explained by strain K population dynamic differences since a density threshold of 10^4 cfu of strain K/cm^2 of apple surface seems to be required just after harvest in order to obtain a high antagonistic activity against *P. expansum* and *B. cinerea* whatever the mode and the time of application. Variations of meteorological conditions between both years of trials are in accordance with population density and efficacy differences observed in case of pre-harvest treatments based on strain K. During the first year

of trial, just one peak of rain was recorded in the orchard during 10 hours with a maximal intensity of 1.4 l/m^2.h (results not shown). In opposite, 4 peaks of more intense rain (up to 5 l/m^2.h) were detected during the second year and could cause washing off of biological but also chemical treatments. Pre-harvest temperatures were ranged between 18 and 25°C during year 2000 while lowest temperature (from 12 to 19°C) were observed during year 2001 and could be less favourable for strain K development. The overall results highlight the necessity to develop a novel formulation, which will take into account strain K adherence, suspension stability and protection against climatic detrimental factors.

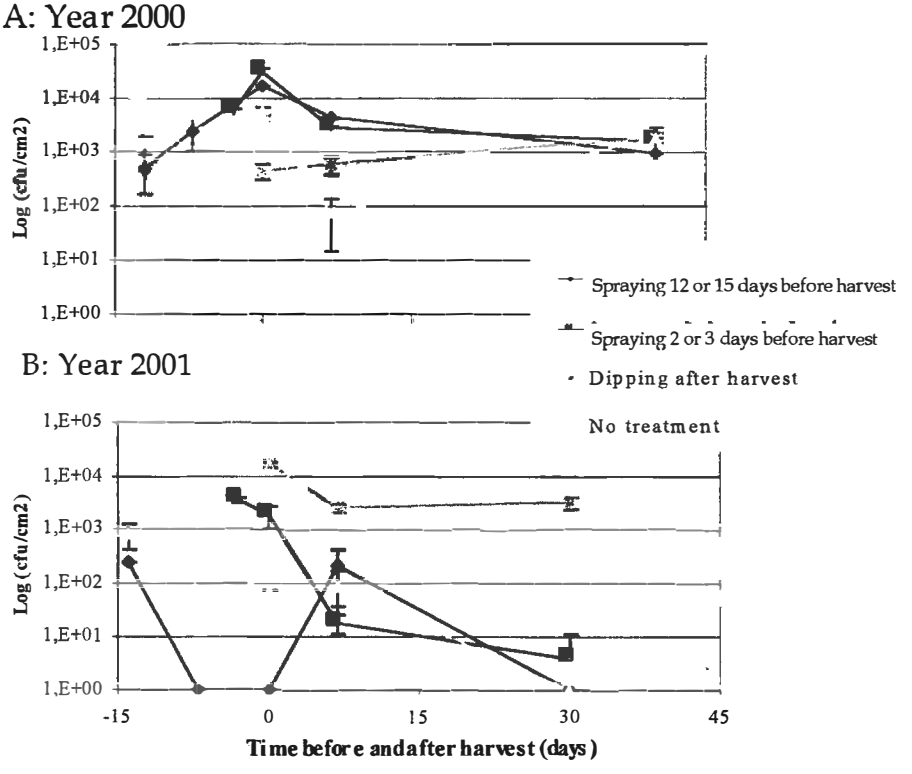

Figure 1: Monitoring of strain K applied in pre-harvest high volume spray 12 or 2 days before harvest during year 2000 (15 or 3 days during year 2001) or by post-harvest dipping. Vertical bars are standard errors of their respective means expressed as log (cfu/cm^2 of apple surface).

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Enhanced efficacy of *Candida sake* CPA-1 by the combination with ammonium molybdate in pre- and postharvest application

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Abstract: Enhancement of biocontrol activity of *Candida sake* (CPA-1) by ammonium molybdate to control postharvest decay in apples and pears was followed during 2 seasons. In preliminary assays the addition of ammonium molybdate 5 mM enhanced the effectiveness of *C. sake* 2×10^6 CFU/ml in controlling major decay on pome fruits. The consistency of these results was maintained in semi-commercial trials. The pre-harvest application of ammonium molybdate did not improve blue mold postharvest biocontrol. Population dynamics of *C. sake* in the presence of ammonium molybdate 5 mM on fruit wounds was also investigated. In both fruits population of *C. sake* significantly decreased in the presence of ammonium molybdate at 20°C and 1°C.

Keywords: postharvest diseases; *Candida sake*; blue mould; grey mould; pre-harvest application

Introduction

Postharvest decay represents major losses in pome fruits. *Penicillium expansum* causing blue mould is the most important postharvest disease, followed by *Botrytis cinerea* causing grey mould and in some cases *Rhizopus stolonifer*. A naturally occurring yeast *Candida sake* CPA-1 isolated from apple surface exhibited activity against the major postharvest diseases of pome fruits (Viñas et al., 1998; Usall et al., 2001). The effectiveness of *C. sake* CPA-1 depends on the concentration of the antagonist (Viñas et al., 1998), thus the manipulation of the environment to promote a rapid increase in *C. sake* population at the wound site or/and to reduce inoculum of the pathogen will probably improve disease control and increase the reliability and efficiency of this biocontrol system. Preliminary studies (Nunes et al., 2001a) showed that the combination of *C. sake* (CPA-1) with ammonium molybdate reduce blue mould decay more effectively than the addition of other nutrients.

The objective of this work was to evaluate the ability of ammonium molybdate (NH_4-MO) to enhance the biocontrol activity of *C. sake* CPA-1 to control major pome fruit postharvest decay.

Material and methods

Laboratory trials under room temperature

Fruits were wounded by removing blocks (3×3×3 mm) at the stem and calyx. In each wound 25 µl of each treatment was applied followed by inoculation of 20 µl of *Penicillium expansum*, *Botrytis cinerea* or *Rhizopus stolonifer* at 10^4 conidia/ml. Treatments were: *C. sake* at 2×10^6 CFU/ml, ammonium molybdate (NH_4-MO) at 1, 5, 10 or 15 mM alone or in

combination with *C. sake*. Since the recommended concentration of *C. sake* is 2×10^7 CFU/ml (Usall et al., 2001), this concentration was used as a prove control. Percentage of wounded fruits (incidence) were measured after 7 days at 20°C and 85±5% RH.

Semi-commercial trials under cold storage

Fruits were wounded in two locations with a nail (1 mm diam. and 2 mm deep), and were dipped in each treatment for 30 s and dipped again in *P. expansum* or *B. cinerea* suspension. Treatments were: *C. sake* 2×10^6 CFU/ml, NH_4-MO at 5 mM and the combination of both. Proves control were: *C. sake* 2×10^7 CFU/ml and the fungicide Imazalil at commercial dose (0.5%). Fruits were stored at 1°C during several months in air and low oxygen atmosphere (LO). This study was conducted during 2 storage seasons.

Postharvest blue mould control by pre-harvest treatments

Field trials were conducted in different ways in apple and pear fruits. Apples were pre-harvest wounded with a nail and treatments were sprayed using a handgun. After 2 days fruits were harvested and sprayed with *P. expansum*. Treatments were: *C. sake* 10^7 CFU/ml, NH_4-MO at 1 mM and the combination of both.

In pears the effectiveness of pre-harvest application of NH4-MO was compared with postharvest application. Pre-harvest application of NH_4-MO 1 mM was applied 2 days before harvest. After harvest pears were wounded and *C. sake* 10^7 CFU/ml was applied. Proves control were postharvest application of: NH_4-MO 1 mM, *C. sake* 10^7 CFU/ml and the combination of both. All treatments were inoculated with *P. expansum*. All fruits were stored at 1°C in air for several months.

Effect of ammonium molybdate on* C. sake *population on fruit

Fruits were wounded as above, dipped for 30 s into the *C. sake* suspension 2×10^6 CFU/ml alone or in combination with NH_4-MO 1 or 5 mM and incubated at 20°C or 1°C in air. To monitor *C. sake* population a known area of pieces of peel surface was removed and shaken in phosphate buffer and sonicated in an ultrasound bath. Serial dilutions were made and plated in nutrient yeast dextrose agar medium. Populations size were expressed as CFU/cm^2 of fruit surface.

Results

Laboratory trials under room temperature

On apples the addition of NH_4-MO at 10 and 15 mM to *C. sake* enhanced in 75% the activity of the antagonist to control blue and grey mould. These combinations allowed a similar or higher control of both pathogens than that obtained with the recommended concentration of *C. sake*. Moreover, against gray mould the addition of NH_4-MO 5 mM to the antagonist enhanced *C. sake* efficiency in 73%. The application of NH_4-MO alone or in combination with *C. sake* completely control *R. stolonifer*. On pears similar results were obtained. The concentration of NH_4-MO at 5 mM was chosen for semi-commercial trials.

Semi-commercial trials under cold storage

All treatments significantly reduced incidence of both pathogens at 1°C in air or in LO (Figure 1). Against blue mould the biocontrol activity of *C. sake* when combined with NH_4-MO was enhanced more than 50% in apples storage in air or in LO atmosphere (Figure 1A) and more than 88% in pears storage in air (Figure 1B). Control of gray mold in apples and pears was more effective with the addition of NH_4-MO to *C. sake* than the antagonist alone at 2×10^6 CFU/ml. In all studied cases the control achieved with the combined treatment was similar or higher than *C. sake* at the recommended concentration or than Imazalil.

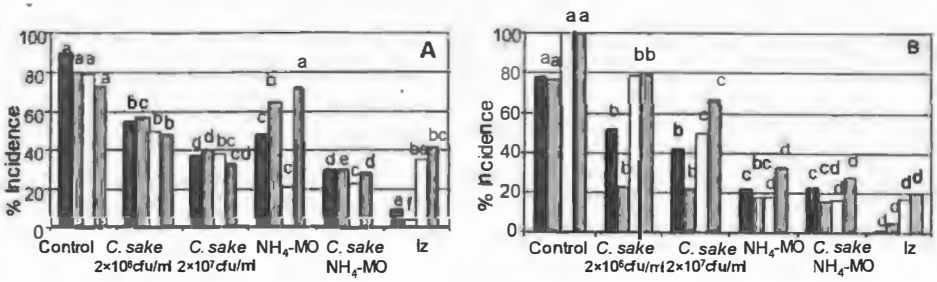

Figure 1. Suppression of decay on (A) Golden Delicious apple and (B) Blanquilla pear stored at 1°C. Treatments were applied followed by inoculation with *P. expansum* and incubation ■ in air or ▥ in LO, or inoculation with *B. cinerea* and incubation ⊡ in air or ▨ in LO. Apples were stored for 4 months and pears for 5 months. Within pathogen and storage, columns with the same letter are not significantly different ($P \leq 0.05$) according to the LSD test.

Postharvest blue mould control by pre-harvest treatments

In pre-harvest wounded apples the combination of *C. sake* 10^7 CFU/ml with NH_4-MO did not improve biocontrol activity, however all treatments significantly reduced blue mold incidence. On pears postharvest wounded, no effect on control blue mold was reported with pre-harvested application of NH_4-MO, and no differences were observed between pre-harvested application of NH_4-MO followed by postharvest application of *C. sake* and postharvest treatment only with *C. sake*.

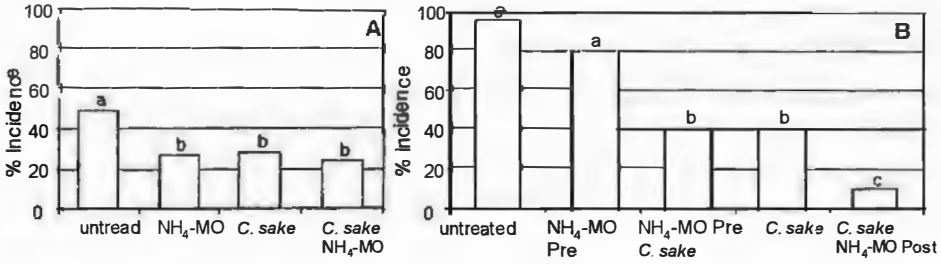

Figure 2. Control of blue mould on fruits pre-harvest treatment with ammonium molybdate and stored at 1°C in air. (A) Apples were wounded and all treatments were applied in pre-harvest; after harvest apples were sprayed with *P. expansum* and stored for 3 months. (B) Pears were pre-harvest treated with ammonium molybdate (NH_4-MO pre); after harvest pears were wounded and treated with *C. sake* or ammonium molybdate (NH_4-MO post). Pears were sprayed with *P. expansum* and stored for 4 months. Columns with the same letter are not significantly different ($P \leq 0.05$) according to the LSD test.

Effect of ammonium molybdate on* C. sake *population on fruit

Ammonium molybdate negatively affect the growth of *C. sake* in apples and pears stored at 20°C and 1°C. No differences were observed in *C. sake* growth in the presence of 1 or 5 mM of ammonium molybdate.

Discussion

Addition of ammonium molybdate to *C. sake* greatly improved the control of the major postharvest disease on apples and pears. These results were maintained in simulating commercial conditions, and in general the combined treatment of *C. sake* 2×10^6 CFU/ml and ammonium molybdate 5 mM conferred a control level similar or higher than the treatment of *C. sake* 2×10^7 CFU/ml alone or imazalil. This means that it is possible to reduce the concentration of the antagonist without diminishing blue and gray mould control.

The application of ammonium molybdate alone in general provides great control. Variability in the performance of the antagonists has been describe as the main problem of biocontrol (Wilson et al., 1996), so this study showed that the combination of *C. sake* with ammonium molybdate could overcome some of the limitation of the biocontrol agent.

The pre-harvest application of ammonium molybdate did not affect blue mold decay. When fruits were pre-harvest wounded and all treatments were applied in the field no differences were observed between ammonium molybdate, *C. sake* and the combination of both. However when pre-harvest application of ammonium molybdate was made in unwounded fruits no effect was observed in reducing blue mold, probably because a residue of ammonium molybdate must remain on the wound to inhibit decay. In other work Smilamick et al. (1999) reported that a residue of sodium carbonate or bicarbonate was necessary to control *Penicillium digitatum* in citrus fruit.

The exact mechanism of action of ammonium molybdate is uncertain. In *in vitro* assays Nunes et al. (2001b) reported the inhibition of spore germination of *P. expansum* and *B. cinerea*. In conclusion the improved control activity of *C. sake* by ammonium molybdate reduce the total biomass of the antagonist needed to ensure effective control. This will be of great value in the implementation of this biocontrol agent.

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Effect of different factors on mass production, formulation and biocontrol activity of the yeast *Pichia onychis* against *Rhizopus stolonifer*, a postharvest pathogen of tomato

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Abstract: Strain Lv027 was selected in a previous study for suppressing *Rhizopus stolonifer* in harvested tomatoes by more than 85% at 6°C and at 22°C. In the present study, factors affecting production, formulation and effectiveness of *P. onychis* Lv027 against *R. stolonifer* were explored. When the interval between yeast application and pathogen inoculation was decreased from 24 hours to 0 hours, effectiveness of control generally decreased from about 94% to 18% at 6°C and from 100% to 0% at 22°C. Application of Lv027 at 6 hours to 24 hours after inoculation with *R. stolonifer* gave little control at 6°C (0-22%) and at 22°C (<2%). Best protection was obtained (88% and 94% respectively) when the yeast was applied 18 hours and 24 hour before at 6°C, and 100% protection in both cases at 22°C. Optimized conditions in liquid fermentation and a biopesticide prototype were obtained.

Key words: *Rhizopus stolonifer*, *Pichia onychis*, tomato, mass production, formulation

Introduction

Biocontrol by means of microbial agents can be an effective alternative to chemical pesticides to control postharvest diseases in fruits and vegetables. In a previous study, the yeast *Pichia onychis* Lv027 strongly suppressed *Botrytis allii* in onion bulbs and *Rhizopus stolonifer* in tomato fruits at 6°C and at 22°C (García et al., 2001). In the present work, effects of application time of the yeast *P. onychis* Lv027 in relation with inoculation time of *R. stolonifer* were examined in tomato fruits, as well as, relationships of inoculum density of Lv027, protection and the effect of nutrient substances and excipients for yeast mass production and formulation.

Materials and methods

To quantify biocontrol activity of Lv027 against *R. stolonifer*, two wounds each 6 mm in diameter and 3 mm deep were made on each of the two groups of tomato fruits. The first group was treated with 25 µl of $1x10^7$ cells/ml yeast suspension and immediately inoculated with a 25 µl suspension of *R. stolonifer* sporangiospores in Tween 80 0.1% containing $1x10^4$ spores/ml. The inoculated tomatoes were incubated in humid chambers at 6°C and 22°C. A second group of tomatoes was inoculated with $1x10^7$ cells ml^{-1} yeast suspension as described above, and incubated 24 hours, 18 hours, 12 hours, and 6 hours before and after the pathogen inoculation (25 µl of $1x10^4$ sporangiospores/ml). Tomatoes were incubated under the same conditions described above. Once disease symptoms appeared in the pathogen control (tomatoes inoculated only with *R. stolonifer*) wound diameter measures were taken every 24 hours from the 3rd day until the 6th day for tomato fruits inoculated at 22°C and every 48 hours

from the 14th day to the 28th day, for tomato fruits incubated at 6°C. Based on disease severity reduction, results were expressed as protection activity (García et al., 2001).

In order to determine the minimal effective concentration for yeast Lv027 against *R. stolonifer* in tomato fruits, five yeast suspensions, with concentrations from 10^5 cells/ml to 10^8 cells/ml were inoculated 24 hours previous to the pathogen inoculation as described above. To optimize a culture medium for *P. onychis*, previously standardized conditions in 125 ml culture media corresponding to level 0 were used: 16 g/l of molasses, 0.0566 g/l hydrolyzed yeast and 0.5 g/l of salts under the following conditions: 150 rpm, 25°C and a ratio of 1/5 of media volume/ flask capacity. Additionally two levels represented by 25% and 50% below the factor 0, and two levels represented by 25% and 50% above the factor 0 were evaluated, by means of 31 treatments. As basis to generate a high quality prediction model, the central composite design was selected, since it can be approached by using a second order polynomial model. Each medium was inoculated to obtain an initial yeast concentration of $1x10^7$ cells ml^{-1}, and incubated during 90 hours. Statistical analysis was applied by using Statgraphics 7.0 with the following codified variables: codified variable = 2 {(Variable – level (0)) / (level (1) – level (-1))}. Each assay was carried out by triplicate. Obtained yield was determined as yeast cell concentration.

In order to obtain a biopesticide prototype, the yeast produced by the fermentation process was characterized by determining its viability, moisture percentage and particle size. The effect of previously characterized excipients on the yeast viability was determined; with the excipients non affecting the yeast viability, biopesticide prototypes were performed, the stability expressed as phases separation time at room temperature allowed to select the most stable prototypes, whose microbiological stability was determined by storing them at 8°C, 20°C and 28°C during one month. The selected biopesticide prototype was stored during three months at the mentioned temperatures and monthly was submitted to physical and microbiological characterization.

Results and discussion

When different intervals between yeast application and pathogen inoculation were evaluated, significant protection was obtained at 6°C only when it was inoculated 24 hours, 18 hours and 12 hours prior to the pathogen inoculation with 94.4%, 87.9% and 77.6% protection respectively; while at 22°C, 100%, 100% and 48% protection was obtained, respectively (Table 2). In contrast, with the other inoculation timing a protection ranged between 30% and 0% was obtained, permitting furthermore, abundant mycelium growth and sporulation of the pathogen in the injured sites, at both temperatures. Similar results were obtained by Jijakli et al., (1993, who attributed this situation to the yeast rapid colonization of wound sites.

When effectiveness of different inoculum densities of the yeast *P. onychis* (Lv027) against *R. stolonifer* was evaluated, a clear dose response was observed. At 6°C, *P. onychis* concentrations of $1x10^8$, $1x10^7$, and $1x10^6$ cells/ml produced the significantly highest protection corresponding to 91.1%, 86.2%, and 82.4% respectively while at 22°C it was obtained when $1x10^8$ cells/ml suspension was applied with 95% protection and no significant differences were observed when tomatoes were inoculated with yeast concentrations between $1x10^7$ and $1x10^4$ cells/ml where protection ranged between 85% and 43%. When protection was inferior to 40% abundant pathogen mycelium growth was produced at both incubation temperatures (Table 3).

Based on the obtained results, for *R. stolonifer* biocontrol $1x10^7$ cells/ml yeast concentration is recommended to be used. These results were similar to the obtained by

McLaughlin et al. (1990), who observed that $1x10^8$ cfu/ml concentration of *Candida* sp. was the most effective for *B. cinerea* and *P. expansum* rot diameter reduction in apple fruits.

Table 2. Biocontrol activity of the yeast *P. onychis* inoculated before, simultaneously and after *R. stolonifer*, in tomato fruits stored during 28 days at 6°C and 6 days at 22°C

	Protection (%)					
Inoculation (hours)	Hours before *R. stolonifer* inoculation		Simultaneously with *R. stolonifer*		Hours after *R. stolonifer* inoculation	
	6°C	22°C	6°C	22°C	6°C	22°C
24	94,4 a	100,0 a			9,8 be	0,7 d
18	87,9 a	100,0 a			22,0 bc	0,3 d
12	77,6 a	48,9 b			8,9 be	1,9 d
6	28,5 b	30,7 c			0,6 c	0,0 d
0			18,0 bc	0,0 d		

Figures in columns followed by a common letter are significantly not different from each other according to the multiple range test LSD ($P \leq 0.05$).

Table 3. Effect of *P. onychis* concentration, on the biocontrol activity against *R. stolonifer*, in tomato fruits stored during 28 days at 6°C and 6 days at 22°C

Incubation temperature	Lesion diameter (mm)				
	Concentration (cells/ml)				
	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$
6°C	43.0b	39.0b	82.4a	86.2a	91.1[a]
22°C	43.0c	60.0cd	72.0bcd	85.1bc	95.0a

Figures in columns followed by a common letter are significantly not different from each other according to the multiple range test LSD ($P \leq 0.05$).

When five levels of every nutrient and agitation factors were evaluated for mass production of *P. onychis* expressed as cells ml^{-1} the following regression model for productivity was obtained:

$$Y = -1.459 + 1.43x10^{-3}X_1 + 10.64x10^{-3}X_2 + 40.88\ X_3 + 0.187X_4 + 1.06x10^{-5}X_1X_2 + 1.58x10^{-3}X_2X_2 - 217.16X_3X_2 - 9.39x10^{-3}X_4X_2 - 3.45x10^{-4}X_1X_2 - 0.0372X_1X_3 + 2.63x10^{-4}X_1X_4 - 0.1325X_2X_3 + 5.95x10^{-4}X_2X_4 - 0.6394X_3X_4.$$

Where X_1, X_2, X_3, X_4 represent the influence of agitation, molasses, nitrogen source and salts respectively, Y represents the final yield (cells/ml) obtained after the fermentative process. The + or - signs represent the positive or negative influence of each factor on the interaction evaluated. This expression that represents a predictive model for *P. onychis* production, was submitted to a maximization process. When obtained optimum values were replaced (Table 4) in the equation 1 it resulted in a predicted yield of $1.01x10^{11}$ cells/ml, in contrast, the basic medium average yields were $5.52x10^{10}$ cells/ml. However when forecast results by mathematics model were validated realizing different experimental runs at different volumes and using the optimum conditions for both nutrient concentrations and shaking, the obtained yield was of $8.9x10^{10}$ cells/ml.

The model determined that for improving the yeast yield, the level of nitrogen source and salts in the media was not significantly different to the proposed in base media (+7.8% and

+1.2% respectively), however the levels of agitation and molasses concentration were reduced (-26.7% and -16.25% respectively).

These results suggest, that only molasses concentration and agitation had an effect in the yeast yield, which can be improved with level –1 of these factors with an increase in productivity of 42% with respect to the non optimized medium.

Table 4. Obtained results for factors used in the optimization mathematical analysis

Factor	Variable Code	Obtained value	Equivalent Level
Agitation	X_1	110.74 r.p.m.	-1
Molasses	X_2	13.42 g/l	-1
Nitrogen source	X_3	0.061 g/l	0
Salts	X_4	0.52 g/l	0

The biopesticide prototype developed in the formulation process consisted of an emulsified concentrate (EC) which is reconstituted in water to form an oil in water emulsion (O/W), selected excipients included a vehicle acting as medium carrier, a suspensor agent, which increased the carrier viscosity and maintained the microorganism particles in suspension avoiding the flocculation and sedimentation processes, and surfactants (lipophilic and hydrophilic) in order to brake the HLB (hydrophilic lipophilic balance) of the fatty medium forming a stable emulsion when the water is added in. The suspension presented physical and microbiological stability during three months storage. The characteristics of the active principle obtained by liquid fermentation were a particle size of 10.62 μM, a humidity of 11.12% and a viability of $26X10^{11}$ cfu/ml.

The obtained results allowed to conclude that different factors affect the yeast biocontrol activity, production and formulation.

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Biocontrol yeasts metabolise the mycotoxin Patulin

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Abstract: We tested 3 efficient biocontrol yeast strains, *Aureobasidium pullulans* (AP), *Cryptococcus laurentii* (CL) and *Rhodotorula glutinis* (RG) for their capability of resisting to and metabolising Patulin. Following *in vitro* incubation, RG was more resistant than CL and AP to high doses of the mycotoxin and yielded the highest rates of Patulin metabolisation. *In vivo*, i.e. in the percentage of apple wounds infected by *Penicillium expansum* even in the presence of treatment with RG, we recorded a 40% decrease of Patulin accumulation in comparison with untreated infected fruits. This finding suggests that residual biocontrol yeast cells present in rotted apples could metabolise or somehow negatively affect Patulin accumulation.

Key words: Patulin, biocontrol yeasts, *Penicillium expansum*, postharvest disease

Introduction

Patulin is a mycotoxin produced by numerous fungi, but mainly by *P. expansum*, a major postharvest pathogen, especially of pome fruits. Patulin has been detected in apples, pears, grapes, plums, cherries, blackcurrants and in derived unfermented juices mainly used as beverages for childhood (IARC, 1986; Larsen et al., 1998; Moss & Long, 2002). Patulin, at high doses, has immunosuppressive properties (Bourdiol et al., 1990), embryo and maternal toxicity in mice, and is genotoxic (FAO/WHO, 1995). No adequate evidence exists about its possible carcinogenicity (IARC, 1986). The highest tolerable Patulin levels in fruit juices have been established in many countries (FAO/WHO, 1996). However, pre and postharvest fungicide treatments and the impossibility of selecting healthy fruits at an industrial level do not ensure desirable absence of contamination in the final produce (FAO/WHO, 2002).

The complete destruction of Patulin occurs during alcoholic fermentation of fruit juices (winemaking and cider making) by enzyme action of the yeast *Saccharomyces cerevisiae* (Harwig et al., 1973; Moss & Long, 2002). Biocontrol yeasts are becoming realistic alternative to fungicides for preventing *P. expansum* rots on stored fruits (Droby, 2001). The aim of this study was to assess the possible *in vitro* and *in vivo* metabolisation of Patulin by 3 biocontrol yeast isolates, AP, CL, and the pink RG protecting stored apples from *P. expansum* infection.

Material and methods

The 3 biocontrol yeasts were grown in triplicates in sterile microtiter plates containing Lilly-Barnett medium with, alternatively, 0, 100, 250, 500 ppm Patulin. Water or 100 ppm Hygromycin B were the controls. Cultures were incubated for 11 days at 22°C, and growth measured as OD at 595 nm in a BioRad Microplate Reader (Lopez-Garcia et al., 2000).

Possible *in vitro* metabolisation of Patulin by the BCAs was assessed on cell free filtrates of the same cultures from growth studies, processed according to AOAC (2000) procedure. Results of HPLC analyses were expressed as percentage of Patulin recoveries.

Antagonistic activity experiments were carried out at 22 °C as described by Castoria et al. (2001), to compare Patulin accumulation in *P. expansum*-infected apples (cv Annurca) treated and untreated with the biocontrol yeast strain RG. Extractions and HPLC analyses (AOAC, 2000) of Patulin were performed when the same severity of infection (rotted lesion diameters) was reached in both treatments.

Results and discussion

Optical density determinations of yeast growth (Figure 1) and HPLC analyses from *in vitro* experiments (Figure 2) showed that strain AP neither significantly resisted to nor efficiently metabolised Patulin, whereas strains RG and CL were both able to grow in its presence and to metabolise the mycotoxin. Strain RG was more efficient than CL in Patulin metabolisation: after 11 days of incubation in the presence of 100, 250 or 500 ppm Patulin, RG determined about 90%, 65% and 53% reductions of mycotoxin concentration, respectively, as compared to the uninoculated controls, in which almost all added Patulin was recovered.

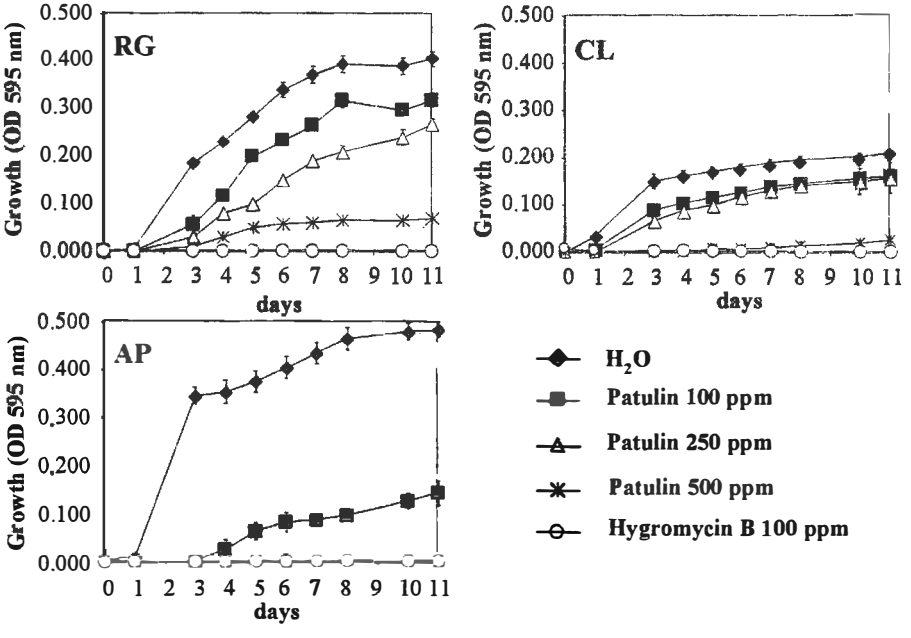

Figure 1. *In vitro* survival (O.D. = 595 nm) at 22 °C of the biocontrol yeasts RG, CL and AP in the presence and 100, 250 and 500 Patulin. Values are the means ± SD of one experiment. Experiments were repeated twice

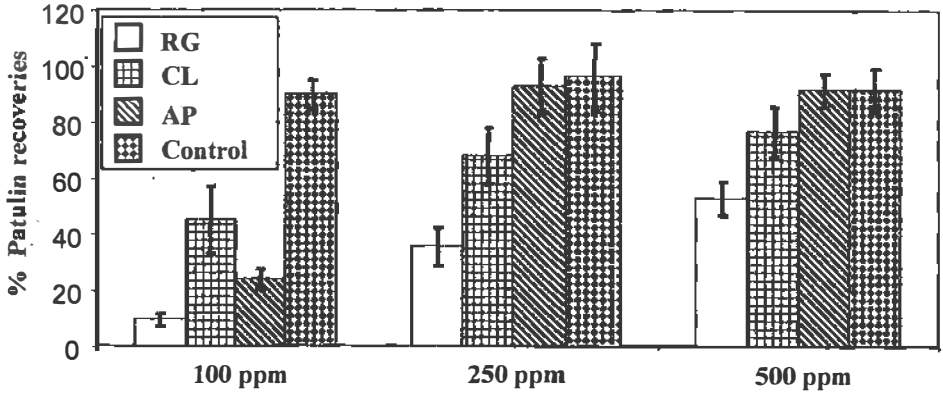

Figure 2. Patulin recoveries from *in vitro* cultures of BCA isolates RG, CL and AP after 11 days of incubation at 22 °C with 100, 250 or 500 ppm mycotoxin. Values are the mean percentages ± SD of one experiment.

A significant reduction of infections (80%) was obtained when wounded apples challenged with *P. expansum* were treated with the biocontrol yeast RG (data not shown). Mean lesion diameters in the percentage of yeast-treated apples infected by the fungus reached the same values as those in untreated infected fruits with a 3 days delay (data not shown).

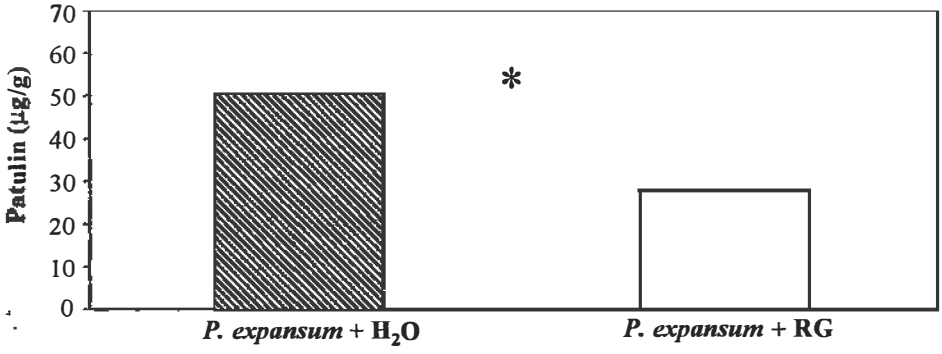

Figure 3. Patulin accumulation in *P. expansum*-infected wounded apples kept at 22° C. Extractions of untreated and RG-treated fruits were performed 4 and 7 days after inoculation of wounds with fungal conidia, respectively, when diameter of lesions reached the same values. (*) Values are the mean of one experiment significantly different at P = 0.01. Experiments were repeated twice.

Further, HPLC analyses showed that Patulin in RG-treated infected apples was about 50% lower than in untreated infected fruits (Figure 3). This indicates that possible residual

RG cells, although not able to prevent infections in a low percentage of wounds, could metabolise or somehow negatively affect Patulin accumulation. We actually recovered only pink yeast cells from rotted apple wounds and AFLP analyses on these cells showed the same electrophoretic pattern as RG strain (data not shown).

The delay in the development of disease symptoms as well as the decrease of Patulin accumulation in biocontrol yeast-treated apples infected by *P. expansum* represent an encouraging result because this **a)** could widen the duration for a safe storage of apples in juice production facilities, **b)** could pave the way for the development of new technologies for the detoxification of Patulin-contaminated juices, **c)** strengthen the role of biocontrol agents in the production of safer foods.

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Biocontrol in soil systems

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Diversity and host specificity of *Pseudomonas* spp. producing the antibiotic 2,4-diacetylphloroglucinol (DAPG)

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Abstract: Biocontrol of soil-borne pathogens has gained enormous interest during the last decades primarily due to public concern about pesticide application. It has been well demonstrated that antibiotics produced by antagonistic bacteria, including 2,4-diacetylphloroglucinol (DAPG), play a key role in the suppression of various of these soil-borne pathogens. Several biotic factors such as the host plant, the pathogen and the density of the producing strain, influence antibiotic production in the rhizosphere and consequently its biocontrol potential. Improvement of the biocontrol potential of a DAPG-producing strain has often been associated with its genotypic characteristics. In general, the genotypic diversity that occurs in natural populations of biocontrol agents provides an enormous resource for improving biocontrol of pathogens. Knowledge of the diversity within a group of indigenous DAPG-producing strains represents a new approach to identify superior biocontrol strains.

Key words: *Pseudomonas*, 2,4-diacetylphloroglucinol, genotypic diversity, biocontrol

Introduction

Antibiotics produced by plant growth-promoting rhizobacteria play a key role in the control of several soil-borne diseases (Thomashow & Weller, 1996). Among these antibiotics are the phloroglucinols that belong to phenolic bacterial and plant metabolites. 2,4-diacetyl-phloroglucinol (DAPG) has been particularly implicated in biocontrol of many plant pathogens by fluorescent *Pseudomonas* spp. Additionally, DAPG-producing *Pseudomonas* spp. have been isolated from the rhizosphere of different crops grown in soils from diverse geographic regions (Keel et al., 1996). Their activity against bacteria, fungi and nematodes has been well documented (Defago, 1993; Keel et al., 1992; Cronin et al., 1997). It is clear that most antagonists, including antibiotic-producing *Pseudomonas* spp., are not consistent in their performance. One of the key factors in the inconsistency of biocontrol is inefficient root colonization of the host plant. The wide variety of organic compounds released by different plant genotypes has been proposed as a key factor influencing the genotypic diversity of microorganisms in different plant spp. rhizospheres (Grayston et al., 1998). This suggests that plant exudate composition is essential in selecting suitable antagonist-plant combinations.

Distribution of indigenous 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.

Many bacterial strains that can produce DAPG have been isolated from a wide range of soils, plant species and geographic areas. Keel et al. (1996) demonstrated that the biosynthetic locus for

DAPG production was highly conserved among 45 DAPG-producing *Pseudomonas* strains of worldwide origin. These two facts support the hypothesis that DAPG producers are an integral part of the indigenous microflora in different soils all over the world. The frequency and distribution of indigenous DAPG-producing *Pseudomonas* spp. in the rhizosphere has gained little attention, so far. The availability of cloned and sequenced genes involved in the biosynthesis of DAPG, has led to the development of specific primers and probes that are routinely used for the detection and isolation of DAPG-producing *Pseudomonas* spp. (Raaijmakers et al., 1997). This has proven to be a powerful technique for studying the ecology of these bacteria in a variety of soils and rhizosphere environments. Colony hybridization followed by PCR analysis has revealed the presence of these bacteria in the rhizosphere of wheat, sugar beet, potato and lily grown in different agricultural soils in The Netherlands. Results obtained so far, indicate that the total population density of the fluorescent *Pseudomonas* spp. in the rhizosphere of the four different plant genotypes ranged from approximately 10^5 to 10^7 cfu/g root. All four crops supported relatively high (10^5 - 10^6 cfu/g root) populations of DAPG-producing *Pseudomonas* spp.

Diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.

The genotypic diversity present in natural populations of DAPG-producing *Pseudomonas* spp. provides an enormous resource for improving biocontrol of soil-borne pathogens (Thomashow & Weller, 1996). Exploitation of this diversity can lead to identification of superior strains with respect to ecological competence and biocontrol potential. Different genotypes of DAPG-producing *Pseudomonas* spp. have been reported to differ in their ability 1) to colonize the wheat rhizosphere and to suppress the take-all disease (Raaijmakers & Weller, 2001), 2) to suppress Fusarium crown and root rot and Pythium root rot (Sharifi-Tehrani et al., 1998), 3) to produce other antibiotics in addition to DAPG (Keel et al. 1996), and 4) to colonize roots of maize plants of different physiological stages (Picard et al., 2000). These examples highlight the ecological significance of genotypic diversity within a group of microorganisms that share the same biocontrol trait. Various DNA-based techniques have been described to determine the genotypic diversity of bacteria, including Random Amplified Polymorphic DNA (RAPD), Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA), Amplified Fragment Length Polymorhism (AFLP) analysis, Denaturing Gradient Gel Electrophoresis (DGGE) (Duineveld et al., 1998), whole-cell BOX- Polymerase Chain Reaction (PCR) and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR (Rademaker et al., 1999) We have used RAPD analysis with three 10-mer primers, which have been shown to provide reproducible banding patterns in *Pseudomonas* (Keel et al., 1996). RAPD analysis requires isolation, selection and purification of the target organism prior to analysis. RAPD-analysis has revealed a higher degree of polymorphism than several other fingerprinting techniques (Mavrodi, 2001; Keel et al., 1996).

Denaturing Gradient Gel Electrophoresis (DGGE) can be used as a culture-free method to determine genetic diversity among bacteria. DNA-fragments of identical size but different sequence composition can be amplified in PCR and resolved by electrophoresis (Theron & Cloete, 2000). In DGGE, separation is based on changes in mobility of DNA fragments migrating in a vertical polyacrylamide gel containing a linearly increasing concentration of DNA denaturants (formamide and/or urea). A sequence of guanines (G) and cytosines (C) is added to the 5`-end of one of the PCR primers (GC-clamp). The GC clamp acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands and allows

detecting nearly 100% of the sequence variants (Muyzer et al., 1998). DGGE is relatively rapid to perform and many samples can be processed simultaneously. So far, most studies on the application of DGGE have focused on the analysis of 16S ribosomal RNA (rRNA) genes to analyze the community structure of microorganisms. In the DGGE-analyses performed in this study, we developed new primers directed against *phl*D, a key gene in the DAPG-biosynthetic locus. The sequence composition of this gene differs among genotypically different DAPG-producing *Pseudomonas* strains (Mavrodi et al., 2001) and therefore may be a useful marker in DGGE analysis. Preliminary genotypic analysis of DAPG-producers, isolated from roots of wheat, sugar beet and potato resulted in 17 different RAPD groups for wheat (N=54), 13 RAPD groups for sugar beet (N=52), and 12 RAPD groups for potato (N=34). DGGE-analysis provided the same overall clustering of the isolates as RAPD analysis, although RAPD analysis provided additional discrimination among isolates within several DGGE groups. For wheat, sugar beet and potato, we identified 6, 6 and 4 DGGE-groups, respectively. Preliminary conclusions that can be drawn, so far, indicate that certain genotypic groups of DAPG-producing strains exhibit a host preference, whereas other genotypic groups occur at relatively high frequencies on multiple plant species. These results open the opportunity to compare the biocontrol efficacy of host and non-host specific DAPG-producers.

Conclusions

There is strong evidence that DAPG producing *Pseudomonas* spp. strains are part of the indigenous microflora in different soils and rhizosphere environments all over the world. Through the use of primers and probes directed against specific sequences within the DAPG biosynthetic locus, we have demonstrated that populations of DAPG producing fluorescent *Pseudomonas* spp. are highly enriched on roots of wheat, sugar beet, potato and lily grown in different agricultural soils in The Netherlands. Different studies have demonstrated that the polymorphism among *phlD* sequences is high. Mavrodi et al. (2001) distinguished 14 different PCR-RFLP clusters using the *phlD* gene among a large collection of DAPG-producing isolates of worldwide origin. They suggested that the *phlD* gene could be a useful marker of genetic diversity and population structure among DAPG producers. In the DGGE-analysis performed in this study, we developed new primers that specifically amplify a 390 bp fragment of the *phlD* gene. Preliminary results indicated a total of seven DGGE groups among the indigenous *phlD*-containing *Pseudomonas* spp. strains tested so far on roots of wheat, sugar beet, and potato. Certain genotypic groups of DAPG-producing strains exhibit a host preference, whereas other genotypic groups occur at relatively high frequencies on multiple plant species.

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The effect of cultural and environmental conditions on the performance of *Trichoderma harzianum* strain T-22

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Abstract: *Trichoderma harzianum* strain T-22 ("T-22") is an US-EPA (Environmental Protection Agency) registered biological fungicide produced and marketed in the U.S.A. by BioWorks Inc. (Geneva, NY, USA) for the prevention and control of different soil-borne and foliar diseases. It is also used commercially for plant growth promotion. The possibilities to register and apply T-22 as a plant strengthener in Europe were investigated. Research was carried out in commercial greenhouses in Spain, France and the Netherlands. From this research it became clear there are many factors that determine the success that can be obtained with T-22. In the Netherlands no plant growth-promoting effect of T-22 was found on the development of vegetable seedlings grown in rockwool. In Spain, however, a positive influence of T-22 on the development of tomato, sweet pepper and celery seedlings grown in peat was observed. On cucumber and tomato plants grown in rockwool in the Netherlands repeated drench applications of T-22 led to 4-10% higher yield only in the case of sub-optimal growing conditions. Nevertheless in *Ficus benjamina*, *Saintpaulia* and Kalanchoe grown under (rather) optimal growing conditions in the Netherlands, higher fresh weights, more flowers and a more uniform plant development was observed. Further research will be carried out to generate more data on the possibilities and limitations of T-22 in order to apply the product in the most effective way under diverse European a-biotic and biotic conditions.

Key words: *Trichoderma harzianum* strain T-22, biological fungicide, plant growth promotion, vegetable crops, ornamental crops

Introduction

Trichoderma harzianum has been under research for decades. It has shown promising results regarding the prevention of soil-borne diseases, but also plant growth promoting effects have been described (Harman, 2000). *T. harzianum* strain T-22 ("T-22"), a patented strain, grows well under a wide temperature range (10-35°C), at a pH between 4-8, in many types of culture media and colonises roots of many types of crops. This EPA registered strain is commercially marketed in the USA since the early 1990's as a biological fungicide (sold under the trade names e.g. PlantShield, RootShield, TurfShield). At this moment this strain is also being marketed as a plant strengthener (trade name TRIANUM) in selected European countries (Spain, Norway and the United Kingdom) by Koppert BV. From 1999 onwards many trials have been carried out with T-22 in the Netherlands, France and Spain to demonstrate the plant strengthening and growth promoting effects of this specific strain under European cultural growing conditions. In this article results of some of the above mentioned trials are presented.

Material and methods

T. harzianum strain T-22 ("T-22") is manufactured by BioWorks, Geneva, NY, USA. Two formulations were tested: a granular formulation (T-22 G), containing approx. $1.5x10^8$ colony forming units ("cfu")/g product, applied by mixing in the growing medium and a suspendable powder (T-22 P) containing approx. $6.0x10^8$ cfu/g and applied by drenching after suspension into water. Nearly all trials were carried out in commercial greenhouses. Only the Dutch trials in *Ficus benjamina* and *Saintpaulia* (both grown in peat) were carried out in small experimental greenhouses. In those trials T-22 P was applied in a dosage of 35 mg/plant in the planting hole just before the rooted cuttings were planted. The plants were destructively analysed 8 weeks after planting. In the Kalanchoe trial the cultivar Sumaco, known for it's difficult root development, was used. T-22 G was manually incorporated into the potting soil at a dosage of 0.75 kg/m³ of growing media. The tomato seedlings of the Dutch trial were sown in rockwool blocks and planted on 7 years old rockwool slabs. T-22 P was applied 4 times at a dose of 35 gram per 1,000 plants. The trials in cucumber were carried out at the sites of 3 different growers (using rockwool or pumice as a substrate) in the Netherlands. Different dosages (from 25-100 g/1,000 plants) and timing (at propagation and/or after planting) of T-22 P applications were tested. In cucumber trials carried out in 2000 and 2001, root samples of T-22 treated and untreated plots in different cultivations by various growers were taken and tested for the presence of different fungi, e.g. *Trichoderma* spp. (identification up to strain level was not possible), *Fusarium oxysporum* and *Penicillium* spp. The trials in Spain were conducted at propagators sites using peat to produce sweet pepper, celery and tomato seedlings in 2 different areas (Almería and Campo de Cartagena). T-22 was applied at different dosages and timings. T-22 G was mixed into the peat at a rate of 0.714-1 g/l peat before sowing. T-22 P was drenched at a rate of 1.5-4 g/m², between the emergence of the cotyledons and transplanting. In the Dutch ornamental and Spanish vegetable trials certain plant growth characteristics (e.g. number of flowers and leaves, fresh and dry weight of roots and shoots, overall plant development) were assessed. The data of the trial in tomato were analysed with ANOVA (95% LSD). In the Dutch vegetable trials the plant growth promoting effect of T-22 was investigated by recording the yield (kg and/or fruits no./ plot).

Results

In sweet pepper higher dry weights were recorded in the T-22 treated plots. The root dry weight of T-22 treated plants was 1.3-1.5 times as high as the root dry weight of untreated plants, while the shoot dry weight of T-22 treated plants was 1.6-1.9 times as high as the shoot dry weight of the untreated plants. T-22 treated plants were also taller than the untreated plants.

The most obvious effect of T-22 on celery seedlings was seen on root development; a much better root system was developed after the application of T-22 (Figure 1). Also the height and number of leaves of the T-22 treated seedlings were greater than untreated seedlings. No differences were found between the 2 dosages (3 or 4 g/m²) of T-22 applied. In Spain the T-22 treated tomato seedlings were significantly ($P \leq 0.0001$) taller than untreated seedlings. No significant difference in shoot dry weight and number of leaves was found. In case T-22 G was mixed through the soil seedlings had a significant ($P \leq 0.05$) lower root dry weight than seedlings from the other treatments. In the Dutch tomato trial a positive effect of T-22 on yield (approx. 10% more yield) was recorded. Until the end of the cultivation *Trichoderma* was found on the

roots of the treated plants. Different plant pathogenic fungi (*Pythium, Fusarium oxysporum* and *Fusarium* spp., *Penicillium* and *Phytophthora*) were detected in both plots, although at the end of production *Pythium* was no longer found in the T-22 treated plot. *F. oxysporum* was not pathogenic on the used cultivar. In cucumber a positive effect of T-22 on yield was observed in all plots treated with T-22; approx. 4% more cucumbers (kg and no. of fruits per plot) were harvested in the treated plots. Root samples taken from plants treated with T-22 showed reduced disease levels in terms of cfu's of various pathogens in comparison to root samples of untreated plants (Table 1).

Figure 1. The effect of T-22 on root development of celery seedlings (U= untreated, T(3) = 3 gram T-22 P/m², T(4) = 4 gram T-22 P/m², both applied just after emergence of the cotyledons).

Table 1. The number of colony forming units of different fungi per gram of fresh root weight of cucumber plants (grown in rockwool).

Cucumber	Sampling Date	Treatment	No. of cfu's per gram of roots (fresh weight)				
			Trichoderma spp.	*Fusarium oxysporum*	*Penicillium* spp.	Others	Total
Grower 1	28-02-	Untreated	6000	15334	0	4000	25334
1st cultivation	2000	T-22	8334	0	0	0	8334
Grower 2	28-02-	Untreated	0	1334	2000	63334	66668
1st cultivation	2000	T-22	4667	0	0	1667	6334

In the *Ficus* and *Saintpaulia* trials T-22 treated plants were more uniform in size and shape than untreated plants. No (positive) influence of T-22 on root development was observed. The T-22 treated *Ficus benjamina* cuttings had a higher percentage dry weight (approx. 10%) and a larger area per leaf, while the number of leaves was the same as of the untreated cuttings. *Saintpaulia* plants showed a better symmetry, developed more leaves and more flowers (nearly 50%!) where T-22 was applied. Kalanchoe plants, treated with T-22 G were taller (24.5 cm) and had more flowering stems (8.1) than untreated plants (23.1 cm and 7.0 flowering stems per plant, respectively) (Figure 2). With the application of T-22 G the fresh and dry weight of plants increased by 15 and 11%, respectively, compared to untreated plants.

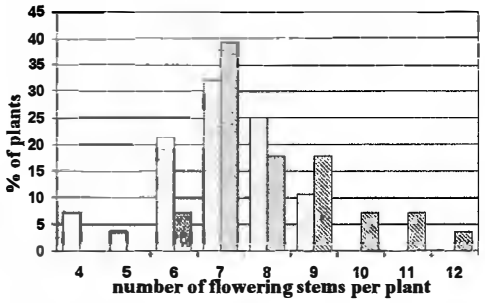

Figure 2. The distribution of flowering stems on untreated (□) and T-22 G treated (▨)Kalanchoe cv. Sumaco plants (n=28). T-22 G was mixed in the growing medium (0.75 kg/m³).

Conclusion and discussion

In all trials in both vegetable and ornamental crops, the plant growth promoting effects of *T. harzianum* strain T-22 were observed. Similar results are also described by Harman (2000), Arena (2000) and Datnoff & Pernezny (1998). Schneller & Albert (2000) found positive effects of T-22 in chrysanthemum, but not in all tested cultivars. Growing medium or environmental conditions may have caused the differences observed when applying T-22 onto peat grown vegetable seedlings in Spain and rockwool grown seedlings in the Netherlands. Van Heemert & Veenstra (1997) observed a stronger effect of T-22 on cucumber seedlings grown in cocopeat than in rockwool. Andreas (unpublished data) found that the type of rockwool can influence the effect of T-22 on cucumber yield. The environmental conditions in Spain are considered to be less optimal for plant growth compared to Dutch conditions. Those sub-optimal conditions cause stress to plants, particularly the root system. This root stress can be minimised by the application of T-22. In all trials disease pressure was low and no plants were lost during the trials. Nevertheless, a suppressing effect of T-22 on the development of other (pathogenic) fungi was observed in the cucumber trials. Harman et al. (1989) observed the same phenomenon of T-22 on *Pythium* spp., *Rhizoctonia* spp. and *Sclerotium* spp. in cucumber.

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Integration of antagonistic bacteria in the process of pelleting sugar beet seed – results and problems

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Abstract: Biocontrol activities at KWS are focused on application of bacterial antagonists (mainly *Bacillus* spp., *Pseudomonas* spp.) mixed into the sugar beet seed pill or into a layer surrounding the pill. Physical properties of the sugar beet pill are not negatively influenced by integration of antagonists. Combination treatments of antagonists and pesticides are generally effective. Moisture content of the soil and temperature during the germination and emergence processes influence activity of antagonists applied to pills. Antagonists may have the potential to accelerate early emergence in the field. Application of antagonists without fungicidal supplementation will result in non maximal final emergence values compared with conventionally pesticide treated seeds. Nevertheless in terms of integrated pest management (diminished concentration of pesticides) and ecological farming application of microbial antagonists holds the perspective to be a reliable component of plant protection and growth promotion. Efficacy persistence in the field is still not satisfying and has to be improved.

Key words: sugar beet seed, pelleting, *Bacillus subtilis*, *Pseudomonas fluorescens*, damping-off, seed emergence, water stress, temperature stress

Introduction

In conventional farming systems phytopathological problems, which might occur during germination and emergence of sugar beet seed, are controlled by seed treatment with fungicides (mainly Thiram, Hymexazol) and insecticides (mainly Imidacloprid, Tefluthrin). For the last years experiments at KWS SAAT AG were performed with the aim to investigate the abilities of microbial antagonists to supplement or replace fungicides. Biocontrol activities at KWS are focused on the application of bacterial antagonists (*Bacillus* spp., *Pseudomonas* spp.) in order to be integrated in conventional and ecological farming respectively. Main aim of application of antagonists is the suppression of pathogenic fungi causing seedling damping-off (mainly *Pythium ultimum*, *Aphanomyces cochlioides*, *Rhizoctonia solani*, *Phoma betae*) to protect germination and emergence processes that are very sensitive. Nevertheless at most of the field trial locations it is not possible to proof a direct suppression of plant pathogens. Therefore counting of emerged seedlings (early and final count) represents the most important parameter for effectivity.

Material and methods

Bacillus subtilis MBI600 (talc based inoculum) and *Pseudomonas fluorescens* B5 (peat based inoculum) were supplied by MicroBio Ltd. Sugar beet seed: seed lot 9-80611/32 (3.25 – 3,75 mm, germinability: 98 %, monogenicity: 99 %, filled fruits: 100 %, thousand-corn-mass: 13.0 g).

Pelleting of sugar beet seeds (0.7 units per batch) were carried out due to KWS standard protocol P-1. Antagonistic microorganisms were pelleted by adding the inoculum to the adhesive. The percentage of the inoculum material to the complete pelleting material differed from 0.1-0.5 %. Antagonistic organisms were mixed into the seed pellet or into a layer surrounding the pill. Pelleting material was examined for the impact on adhesion and survival of antagonistic bacteria. Additionally pills applied with antagonists were investigated for effects on physical (e.g. hardness, roundness) and biological (e.g. germinability, vigour) characteristics of the pill. Freshly prepared pills have a moisture content of 35 to 50 %, which is reduced by the drying process to a target value of 8-9%. The drying process was carried out due to KWS standard protocol D-1.

Emergence test in natural soil and field trials

An emergence-test in natural soil were performed to KWS standard protocol E-1: kinetics of seed emergence were created from early (optimal conditions: 4 days after drilling, stress conditions: 7 days) to final emergence (optimal conditions: 7 days after drilling, stress conditions: 10 days). In collaboration with KWS Italia and KWS France field trials were performed in spring 2001. Field trials in Italy were carried out in Monselice located close to Padova in the region of Veneto while the trials in France were sown in Buzet sur Baise, 100 km south-west of Bordeaux (Table 1).

Table 1. Field trials 2001: Monselice / Italy, Buzet / France

	Monselice / Italy	Buzet sur Baise / France
soil texture	sandy clay loam	sandy loam
infestation with pathogen	light inf. with *P. ultimum*	heavy inf. with *R. solani*
design of the trial	randomized block design, 4 replicates, 3 rows/ plot 300 seeds/ plot	randomized block design, 4 replicates, 6 rows/ plot, 300 seeds/plot
distance of seeds	6 cm	6 cm
drilling depth	2-3 cm	2-3 cm

Results

Emergence of sugar beet seed in natural soil in a climatic chamber (Figure 1) revealed that water and temperature stress (soil temperature 13 °C, soil matric potential 2 kPa, compared to optimal conditions: 20 °C, 20 kPa) caused a delay of emergence by 3 days and a decrease of the absolute level of emergence. There is the tendency that application of antagonists promotes early emergence (optimal conditions, counting after 4 days). *Bacillus subtilis*, strain MBI600, seems to reduce the negative effects on emergence faced to stress conditions. Trial in Monselice / Italy (Table 2): Early countings of sugar beet emergence indicated that emergence increased when pesticides were not applied to the pill. All pills containing bacterial antagonists had higher emergence rates than their corresponding checks without pesticides or with reduced fungicide load. Best results were achieved when isolate *Pseudomonas fluorescens* B5 was applied solely. Although final emergence was highest for pills supplemented with commercial concentrations of fungicides and Imdidacloprid pills solely treated with isolate B5 were not significantly different. Trial in Buzet / France (Table 2): Highest emergence at the early counting date was recorded for the commercial check, followed by all variants with reduced fungicide concentrations and

variants without pesticides. Nevertheless statistical analysis revealed only two variants (B5, MBI600+B5, each without pesticides) to be significantly different. Pure application of MBI600 and MBI600+B5 resulted in significantly lower final emergence.

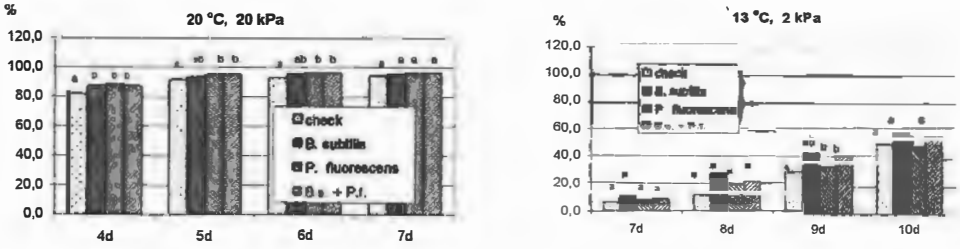

Figure 1: Emergence test in natural soil: influence of temperature and moisture content of soil on emergence of sugar beet pills, applied with different antagonists (*B. subtilis*, isolate MBI600; *P. fluorescens*, isolate B5; mixture of MBI600 and B5) without pesticide.

Discussion

The only proof of concept which could be regarded as a base for a commercial application of biocontrol agents are repeatable control effects in the field. In this context results of field trials performed in 2001 demonstrated that a heavy infestation with pathogens (mainly *Rhizoctonia solani* and *Aphanomyces cochlioides*) could not be controlled successfully with a single application of bacterial antagonists but that biocontrol seemed to work when lower infestation levels (e.g. with *Pythium ultimum*) were present. Due to these facts maximal pathogen control in combination with maximal final emergence could not be achieved without fungicides. Nevertheless a combination of tolerant varieties, higher amounts of seeds for drilling and a non-chemical seed treatment will result in a feasible and commercially acceptable application for eco-farming. An integrated approach of sugar beet seed treatment – application of antagonists combined with diminished amounts of fungicide – seems to be a way for practical sugar beet growing with a minimal risk for the conventional farmer. The most challenging task for the future will be an improvement of efficacy persistence in the field. The variation of results - concerning pathogen control and impact on emergence - remains as the hurdle, which hinders a broader commercial application.

Acknowledgements

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Table 2: Field trials 2001: emergence, early and final count.

Monselice / I: early count

No	antagonist	T	H	I	x abs	
4	B5	-	-	-	56.3	a
5	MBI600 + B5	-	-	-	53.0	ab
3	MBI600	-	-	-	49.7	abc
2	-	-	-	-	48.1	abc
1	-	1	1	1	46.6	abc
8	B5	0.5	0.5	1	46.3	abc
7	MBI600	0.5	0.5	1	44.7	bc
9	MBI600 + B5	0.5	0.5	1	42.7	bc
6	-	0.5	0.5	1	39.5	c

final count

No	antagonist	T	H	I	x abs	
1	-	1	1	1	89.1	a
9	MBI600 + B5	0.5	0.5	1	86.6	ab
8	B5	0.5	0.5	1	85.6	abc
7	MBI600	0.5	0.5	1	84.3	abc
6	-	0.5	0.5	1	83.5	abc
4	B5	-	-	-	82.8	abc
5	MBI600 + B5	-	-	-	80.6	bc
3	MBI600	-	-	-	78.8	bc
2	-	-	-	-	77.3	c

Buzet / F: early count

No	antagonist	T	H	I	x abs	
1	-	1	1	1	51.3	a
7	MBI600	0.5	0.5	1	46.9	ab
9	MBI600 + B5	0.5	0.5	1	46.3	ab
8	B5	0.5	0.5	1	40.8	ab
6	-	0.5	0.5	1	40.0	ab
4	B5	-	-	-	37.8	ab
2	-	-	-	-	36.1	ab
3	MBI600	-	-	-	34.4	b
5	MBI600 + B5	-	-	-	32.5	b

final count

No	antagonist	T	H	I	x abs	
1	-	1	1	1	78.0	a
8	B5	0.5	0.5	1	73.9	a
7	MBI600	0.5	0.5	1	73.8	a
9	MBI600 + B5	0.5	0.5	1	73.8	a
6	-	0.5	0.5	1	65.1	ab
2	-	-	-	-	54.8	bc
3	MBI600	-	-	-	48.9	bc
4	B5	-	-	-	47.3	c
5	MBI600 + B5	-	-	-	44.3	c

T = Thiram, H = Hymexazol, I = Imidacloprid; 1 = normal concentration (T = 11g/U, H = 14.7 g/U, I = 90g/U), 0.5 = ½ normal concentration; values that do not differ significantly ($p>0.05$) share the same letter

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Pythium oligandrum as biocontrol agent of *Phytophthora cryptogea*

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Abstract: Relationship between *Pythium oligandrum* oospores number, incubation time and population dynamic of the mycoparasite and *Phytophthora cryptogea* were evaluated. Additionally, development of *Phytophthora* foot rot of gerbera in peat amended with *P. oligandrum* was studied. Amendment of peat with *P. oligandrum* resulted in the increase of population density of the mycoparasite during 3-4 month-incubation. *P. oligandrum* applied at doses 50 and 100 oospores/g of peat, but especially in the highest amount, caused the decrease of *P. cryptogea* population density. The mycoparasite at dose 100 oospores/g, mixed with peat 10 days before gerbera planting, effectively controlled *Phytophthora* foot rot development of gerbera.

Key words: *Pythium oligandrum*, *Phytophthora cryptogea*, population, gerbera, control

Introduction

Over a past 20 years some new, biocontrol products has been introduced for some plant pathogens control, including *Phytophthora cryptogea* Pethybr. et Laff. The pathogen is the causal agent of *Phytophthora* rot of several plants grown under covering and in the open field. In greenhouse production of gerbera (*Gerbera jamesonii* Bolus) *P. cryptogea* is the main dangerous threat causing *Phytophthora* foot rot. Application of *Pythium oligandrum* Drechsler as gerbera drench resulted in suppression of disease development (Orlikowski, 2001). Mechanism by which *P. oligandrum* operates differs according to the host species (Benhamou et al., 1999). All pathogenic *Oomycetes* and true fungi are highly vulnerable to an attack by *P. oligandrum*. At least 2 distinct mechanisms are involved in the process of fungal attack by *P. oligandrum*: mycoparasitism, mediated by intimate hyphal interactions and antibiosis, with alteration of the host hyphae prior to contact with the antagonist (Benhamou et al., 1999). Reaction of host on the mycoparasite may be cytoplasm aggregation followed by necrotization of hyphae and extrusion of cell content, rapid lysis of the host cytoplasm and cell wall disruption or/and induction of a host structural response at potential entry sites for the antagonist. In case of *Phytophthora megasperma* (Kleb.) Kleb., Benhamou et al. (1999) found that production of toxic compounds by *P. oligandrum* resulted in disorganisation of the pathogen hyphae in the absence of physical contact with the antagonist. In this study relationship between *P. oligandrum* oospores number mixed with substratum, incubation time and population dynamics of the mycoparasite and *P. cryptogea* in substratum were evaluated. Additionally, influence of the mycoparasite on healthiness of gerbera was studied.

Materials and methods

Polyversum, containing 10^6 oospores/g was used. The product was applied at dose 10, 50 and 100 oospores/g of peat. Oospores were mixed with the substratum 10 days before and immediately before gerbera planting. *Phytophthora cryptogea* - The isolate G80 obtained from rotted foot of gerbera was used. The culture was maintained on potato-dextrose agar at 24 °C in the dark. For peat infestation the culture was grown on Quick rolled oats. After 2-week incubation culture was blended with minimum of distilled water in a Waring Blender to form a thick slurry. The homogenate was mixed with peat and substratum was stored in plastic bags with holes at 20 °C. For estimation of population densities of *P. cryptogea* and *P. oligandrum* gallic acid selective medium and procedure of Flowers and Hendrix (1969) was used. Number of colony forming units/g of air-dry peat was estimated at weekly intervals. For testing the biological activity of *P. oligandrum* in the control of *Phytophthora* foot rot of gerbera plants grown on greenhouse bench in the infested peat amended with *P. oligandrum* oospores and without the antagonist (control) were observed weekly and number of wilted plants were noticed. Experimental design was completely randomised with 4 replications and 1 Petri dish or 10 plants in each rep. Trials were repeated at least twice.

Results and discussion

***Population dynamic of* Pythium oligandrum *in peat infested with* Phytophthora cryptogea**

Initial oospores number had significant influence on population dynamic of *Pythium oligandrum* in the substratum (Figure 1). When peat was amended with 10 or 50 oospores/g population density of the mycoparasite increased very slowly within 2-week-incubation and after the next 1 week reached the levels 370 and 430, respectively. During the next 2 weeks number of colony forming units of the mycoparasite decreased (Figure 1).

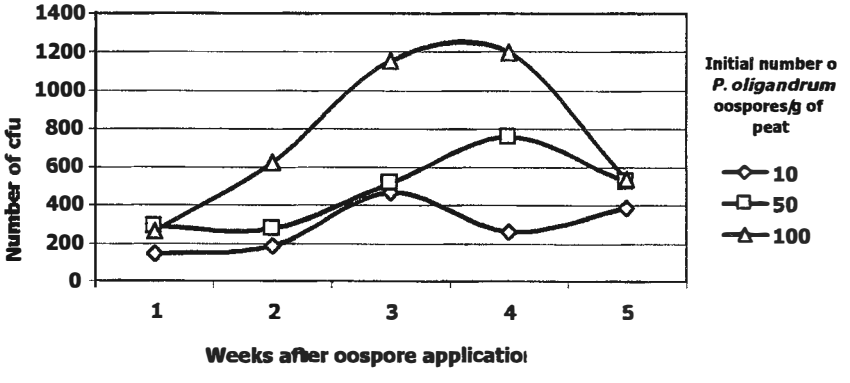

Figure 1. Relationship between initial oospores number of *Pythium oligandrum* mixed with peat, incubation time and population dynamic of the mycoparasite.

***Population dynamic of* Phytophthora cryptogea *in peat amended with* Pythium oligandrum**
In nontreated peat population density of *P. cryptogea* increased during the first 3 weeks of incubation and after the next 14 days reached a level about 250 colony forming units (cfu)/g (Figure 2). Application of 10 oospores of *P. oligandrum*/g of peat resulted in the increase of the pathogen population density within 3 weeks. During the next 2 weeks the number of cfu significantly decreased. Increase of the mycoparasite dose to 50 oospores/g resulted in the suppression of the pathogen development during 5-week-incubation (Figure 2). When the mycoparasite was applied at the highest dose, a population density of *P. cryptogea* increased within first weeks but later significantly decreased (Figure 2).

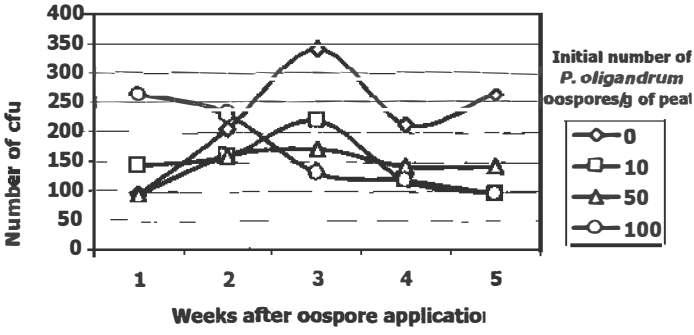

Figure 2. Population dynamic of *Phytophthora cryptogea* in relation to initial *Pythium oligandrum* oospores number in peat and incubation time

Biological activity of* P. oligandrum *in the control of Phytophthora foot rot of gerbera
First, sporadically occurred disease symptoms on gerbera were observed after 4-week-growth. After 7 weeks significantly less plants with *Phytophthora* foot rot were observed when gerbera was grown in peat amended with *P. oligandrum* oospores 10 days before planting (Figure 3A). Two weeks later about 55% of control plants and treated with 50 oospores/g of gerberas showed *Phytophthora* foot rot symptoms. Application of *P. oligandrum* at dose 100 oospores/g suppressed the disease spread about twice (Figure 3A). Amendment of peat with *P. oligandrum* immediately before gerbera planting did not inhibit the disease development. (Figure 3B).

Studies of relationship between initial oospores number of *P. oligandrum* and population dynamic of *P. cryptogea* in peat during 5 weeks showed a correlation between the mycoparasite dose and development of the pathogen in substratum. The mycoparasite propagule numbers in amended peat increased during the first 3-4-week-incubation. Application of the mycoparasite at dose 50 or 100 oospores/g strongly suppressed the pathogen development in peat. Studying of biological activity of *P. oligandrum* in the control of *Phytophthora* foot rot of gerbera showed that at least 100 oospores/g of substratum were necessary for effective control of the pathogen. Results obtained indicated that period of the mycoparasite application played an important role in the disease control. Within 10 day after amendment of peat with oospores the mycoparasite had ability to establish in substratum and operated against *P. cryptogea*. In our *in vitro* study we did not observe a coiling and hyphal invasion of *P. cryptogea* by *P. oligandrum* (Orlikowski & Jaworska-Marosz, unpubl.).

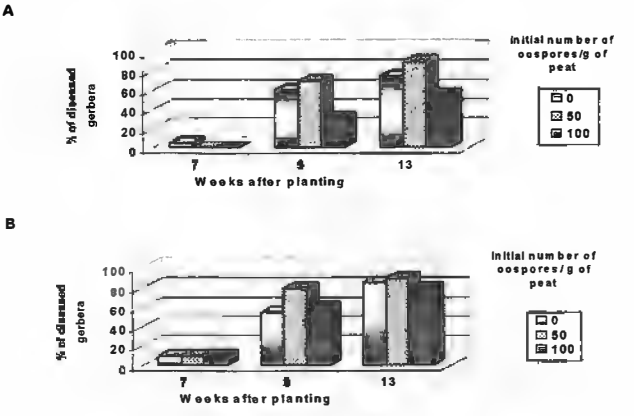

Figure 3. Relationship between oospores number of *Pythium oligandrum* mixed with peat 10 days before (A) and immediately prior to planting (B), growing period and development of *Phytophthora* foot rot of gerbera

This confirms results obtained by Benhamou et al. (1999) with the interaction between *P. oligandrum* and *P. megasperma*. The authors found, however, typical disturbances such as retraction and aggregation of the host cytoplasm as the result of diffusible compounds released by the mycoparasite. Picard et al. (2002) found that a low - molecular weight protein, termed oligandrin, applied to decapitated tomato displayed the ability to induce plant defence reactions that contributed to restrict stem cell invasion by *Phytophthora parasitica*. In tomato stems from oligandrin-treated plants, restriction of *P. parasitica* growth to the outermost tissues and decrease in pathogen viability were the main features to the host-pathogen interaction. It is possible that similar interaction between *P. oligandrum* and *P. cryptogea* may occur in gerberas.

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Biocontrol of root diseases of fruit trees with dsRNA – merit and perspective

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Abstract: Violet root rot of apple trees caused by *Helicobasidium mompa* and white root rot of Japanese pear trees and grapevine caused by *Rosellinia necatrix* are difficult to control without extensive, biennial application of chemicals into soil. Biocontrol with dsRNA is considered applicable to these diseases since 1) the biocontrol agent persists and spreads through the network of fungal mycelia in the soil, 2) biocontrol with dsRNA is environmentally safer, and 3) it pays to apply to fruit trees which are grown with intensive cultural practices in Japan. Surveys on population structure of the pathogens based on mycelial compatibility (MC) revealed that diseased trees had a single MC group (MCG) and that each MCG existed in distinct disease patches. dsRNA was detected from 76.3% and 19.9% MCGs of of *H. mompa* and *R. necatrix*, respectively. Most MCGs with dsRNA were virulent. Electrophoresis with hyphal tip isolates from weakly virulent strains of *R. necatrix* with dsRNA failed to detect dsRNA, and they restored virulence. Further studies are necessary to develop transfection methods of dsRNA to different MCGs and to minimize the interference by indigenous dsRNA.

Key words: *Helicobasidium mompa*, *Rosellinia necatrix*, violet root rot, white root rot, fruit trees, dsRNA

Introduction

Helicobasidium mompa Tanaka and *Resellinia necatrix* Prillieux cause violet and white root rot of various crops, respectively. Intensive cultural practices, such as the use of dwarf stock, glasshouse cultivation, etc., predispose plants to the diseases. The diseases can be controlled only by biennial drench of 50–100L of chemicals for each tree. Biocontrol with soil microorganisms proved ineffective under field conditions. Long-term control may be hampered by the perennial growth of hosts and by the difficulty in the establishment of antagonists in soil. Crop rotation or soil amendment is not applicable, either. Fungal viruses with dsRNA genome (Buck, 1986) are promising against root diseases of fruit trees since they exist within the cytoplasm of fungal hyphae and need no effort to help them persist in the field. The viruses are considered to spread though the network of fungal mycelia in the soil once they enter the fungal cytoplasm. Here, we present preliminary results from a project to control the root diseases of fruit trees with dsRNA (Matsumoto, 1998).

Materials and methods

Isolates of *H. mompa* and *R. necatrix* were obtained from diseased roots and fruit bodies (Nakamura et al., 2001) They were paired on oatmeal agar plates to determine mycelial compatibility groups (MCG). Two isolates producing a dark demarcation line between colonies were assigned to different MCGs. Hyphal anastomosis between them resulted in the death of fused cells. Population structure of the pathogens were determined based on MCG.

Isolation and electrophoresis of dsRNA followed the method of Arakawa et al. (2002). DNA and single-stranded nucleic acids were digested with DNase and S1 nuclease. Samples were electrophoresed on 1.5 % agarose gel. Isolates containing dsRNA were denoted as dsRNA positive. The effect of dsRNA on virulence in *R. necatrix* was studied between dsRNA-negative isolates and their parental, dsRNA-positive strains. dsRNA-negative isolates were obtained by hyphal tip isolation from weakly virulent strains. Two-week-old seedlings of *Lupinus luteus* were inoculated with an inoculum twig fragment at the base and incubated for 2 weeks (Uetake, 2001). The rate of killed plants was the criterion for virulence.

Results and discussion

Six-11 isolates for each diseased trees were first obtained from underground parts. Pairing in all possible combinations within each tree revealed that, with an exception of a tree with white root rot located on the junction of disease patches, isolates from the same trees belonged to the same MCGs in both *H. mompa* and *R. necatrix*. Subsequent analysis on population structure indicated that each orchard had a few disease patches which were occupied by single MCGs. Large patches occupied by prevalent MCGs of *H. mompa* spread more extensively than those with less prevalent MCGs in 4 years. These observations indicate that, on starting biocontrol, one should determine which patch to control since inoculum containing dsRNA is custom-made to apply to specific MCGs.

A variety of dsRNA elements differing in number and size was detected from 464 out of 608 MCGs (76.3%) for *H. mompa* and 82 out of 413 MCGs (19.9%) for *R. necatrix*. Isolates of the same MCGs from single patches often had different dsRNAs. These findings may not preclude the possibility that indigenous dsRNAs interference with novel dsRNAs introduced as biocontrol agents and reduce their effect. Fitness of *R. necatrix* MCGs was compared in terms of virulence, competitive saprophytic ablity, and the presence or absence of dsRNA. MCGs that were found both from roots and soil were generally more virulent than satellite MCGs found exclusively from soil. Satellite MCGs tended to be less competitive for colonized subtrate and to have dsRNA.

Hyphal tip isolation was made from weakly virulent strains of *R. necatrix* with dsRNA to remove dsRNA. Whereas, less than 20% of plants were killed 14 days after inoculation with a parental strain (W370), its hyphal tip isolates without dsRNA (W370f-1 and f-2) recovered virulence, killing almost 100% of plants (Figure 1). Hyphal tip isolation to recover virulence was successful in some other strains. dsRNA is transmitted through hyphal anastomosis between somatically compatible strains, and simple population structure of pathogens is prone to the dissemination of dsRNA. Surveys on population structure of the two soilborne pathogens of fruit trees demonstrated the feasibility of biocontrol with dsRNA along with the economic value of individual trees. Methods to transfect dsRNA to different MCGs should be developed so that any

MCGs may be infected. Our ultimate goal is to breed strains that are compatible with any MCG and to formulize them for field application.

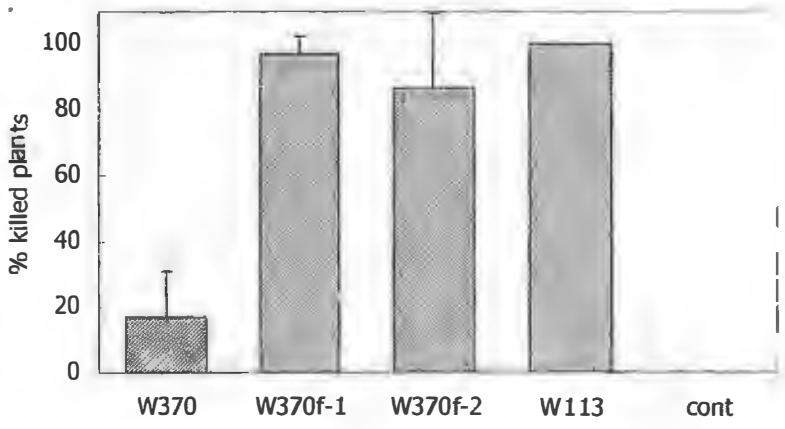

Figure 1. Recovery of virulence by hyphal tip isolation. W370: original strain with dsRNA; W370f-1and F-2: dsRNA-free, hyphal tip isolates from W370; and W113: virulent control isolate without dsRNA

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Microclimate and biocontrol

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Effect of environmental factors on conidial germination of the *Botrytis* spp. antagonist *Ulocladium atrum*

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Abstract: The antagonistic fungus *Ulocladium atrum* can compete on necrotic plant tissues with necrotrophic *Botrytis* spp. Application of *U. atrum* in biocontrol is aimed at a slower inoculum build-up by *Botrytis* spp. in crops resulting in less severe epidemics. Necrotic aboveground plant tissues are characterised by low nutrient content and micro-climatic conditions often unfavourable for microbial growth. In such an environment, success of an antagonist depends on its ability to (1) germinate at low nutrient levels, (2) survive UV radiation, (3) germinate and colonise rapidly within few hours during wetness periods, often combined with low temperatures, and (4) survive frequent dry periods, often combined with high temperatures. Various experiments were carried out to investigate the effect of temperature, nutrient level, UV radiation and water availability on conidial germination and survival of *U. atrum*. From the experimental results it can be concluded that *U. atrum* is adapted to the harsh environmental conditions of above-ground necrotic tissues. Conidia do not depend on exogenous nutrients, can germinate during the short wetness periods at a broad temperature range and survive unfavourable periods with high UV radiation, high temperatures and dryness.

Key words: *Botrytis cinerea*, conidia, competitive colonisation, micro-climate, *Ulocladium atrum*

Introduction

The antagonistic fungus *Ulocladium atrum* can compete on necrotic plant tissues with necrotrophic *Botrytis* spp. Application of *U. atrum* in biocontrol is aimed at a slower inoculum build-up by *Botrytis* spp. in crops resulting in less severe epidemics. This has been demonstrated successfully in several crops such as grapevine, strawberry and cyclamen (Schoene & Köhl, 1999; Köhl et al, 1998, 2000; Boff et al., 2002).

Necrotic aboveground plant tissues as the target for biocontrol with *U. atrum* are characterised by low soluble nutrient content and micro-climatic conditions often unfavourable for microbial growth, e.g. temperatures, water availability and UV radiation can fluctuate rapidly (Diem, 1971; Park, 1982; Hjeljord et al., 2000). Under conditions of Dutch summers, the mean duration of wetness periods in above ground necrotic onion leaf tissue was 4.9 h per day with a mean temperature of 14.8 °C (Köhl et al., 1999). In such an environment, success of an antagonist depends on its ability to (1) germinate at low nutrient levels, (2) survive UV radiation, (3) germinate and colonise rapidly within few hours during wetness periods, often combined with low temperatures, and (4) survive frequent dry periods, often combined with high temperatures. Various experiments were carried out to investigate conidial germination and survival of *U. atrum* under such harsh environmental conditions.

Material and methods

Nutrient level and temperature
Conidal suspensions of *U. atrum* were sprayed on glass slides covered by a thin layer of aga or on sterile necrotic lily leaves, which were subsequently incubated under moist conditions at various temperatures between 3 and 36 °C (Köhl et al., 1999). Percentage germinated conidia was determined in regular time intervals during incubation. From the cumulative percentage of germinated conidia the time needed for 50% germination was estimated per temperature and substrate.

UV radiation
Conida of *U. atrum* were sprayed in an onion crop on hot, sunny days with maximum temperatures above 30 °C at bright sunshine (11:30) or after sunset. Leaf samples were collected during the consecutive days and conidial germination was assessed directly after sampling or after additional incubation in moist chamber.

Water availability
Conidial suspensions of *U. atrum* were sprayed on glass slides covered with a thin layer of water agar. Glass slides were incubated in sealed Petri dishes containing different glycerol-water mixtures to obtain various water potentials (Köhl & Molhoek, 2001). Experiments were carried out with constant water potentials or alternating water potentials. To alternate water potentials glass slides were transferred to different Petri dishes containing different glycerol-water mixtures.

Survival on leaf surfaces
Conidial suspensions of *U. atrum* were sprayed on cyclamen crops grown in commercial greenhouses with or without top-irrigation (Köhl et al., 1998). Leaves were sampled at weekly intervals during ten weeks. The percentage germinated conidia of *U. atrum* was quantified microscopically directly or after additional incubation in moist chamber.

Results

Temperature and nutrient level
Conidia of *U. atrum* germinated very rapidly - within a few hours - at a broad temperature range. A level of 50% germinated conidia was reached within 2.6 h under optimum conditions and even at 6 °C only 18 h were needed to reach this level of germination. The cumulative germination of conidia was the same for conidia incubated on water agar or on necrotic lily leaves. Germination was not enhanced when conidia were incubated on nutrient agar (1/10 malt agar).

UV radiation
During three days after applications of conidial suspensions of *U. atrum* to field grown onions no germination of the *U. atrum* conidia was observed on leaves assessed directly after sampling since hot and dry weather did not support fungal development. On all leaf samples incubated in moist chamber after sampling, the percentage of germinated conidia was above 80% (Figure 1). No differences were found in germinability of conidia exposed after spraying to bright sunlight and those that had been sprayed after sunset.

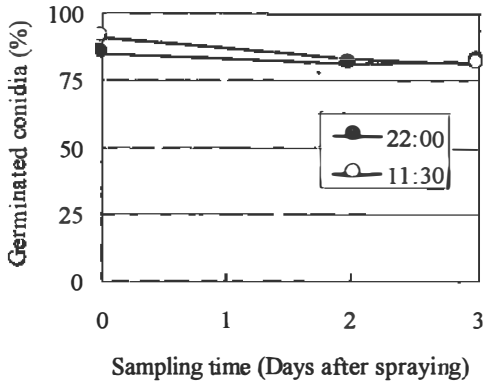

Figure 1. Percentage germinated conidia of *U. atrum* sprayed at bright sunlight (11:30) or after sunset (22:00) in an onion crop. Leaf samples were incubated in moist chamber and spore germination observed microscopically.

Water availability

At high water potential (–0.28 MPa), conidia germinated rapidly within 7 h and reached a maximum of 91% germinated conidia. Speed of germination and the maximum percentage germinated conidia decreased with decreasing water potential. At –7 MPa, conidia needed more than 24 h to reach a maximum of 41% germinated conidia. No germination was observed at –10 MPa. Under field conditions, water availability is mostly fluctuating. The effect of rapid fluctuations on the development of *U. atrum* germlings was investigated under controlled conditions. Interruption of moist incubation by a dry period initiated after 0 to 6 h of moist incubation, did not affect the maximum percentage of germinated conidia (Table 1). Germlings of *U. atrum* survived multiple dry-wet cycles, e.g. wet periods of 6 h followed by dry periods of 18 h. Germ tube development continued during each wetness period without significant lag time.

Table 1. Maximum percentage germinated conidia of *U. atrum* on glass slides incubated under moist conditions interrupted by a dry period (Data from Köhl & Molhoek, 2001)

Duration of initial moist period [2]	% Germinated conidia[1] after dry period at –10 MPa	–42 MPa
0	91	88
2	85	90
4	87	91
6	90	88

[1] Parameter estimates from Gompertz curves fitted to observed values.
[2] Sum of hours of initial moist period and dry period was 24 h.

Survival on leaf surfaces

Observations of cyclamen leaves sampled during ten weeks after application of conidial suspensions of *U. atrum* indicated that only a fraction of the conidia germinated on the leaf surfaces under greenhouse conditions. After additional incubation in moist chamber, 70 to 95% of the conidia had developed germ tubes. After induction of necrosis, *U. atrum* was able to successfully exclude *B. cinerea* from leaf tissue even when conidia had been sprayed 10 weeks before induction of necrosis.

Discussion

From the experimental results it can be concluded that *U. atrum* is adapted to the harsh environmental conditions of above-ground necrotic tissues. Conidia do not depend on exogenous nutrients, can germinate during the short wetness periods at a broad temperature range and survive unfavourable periods with high UV radiation, high temperatures and dryness. These characteristics make *U. atrum* an attractive candidate as BCA for control of *Botrytis* spp.

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Effect of greenhouse climate on biocontrol of powdery mildew (*Leveillula taurica*) in sweet pepper and prospects for integrated disease management

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Abstract: Powdery mildew (*Leveillula taurica*) severely attacks sweet pepper during the whole year. Yellowish spots appear on the upper surface of the leaves and the powdery mildew pathogen appears on the under or, sometimes, the upper surface of the leaves. Infected leaves are shed and the fruit yield quantity and quality fall. The interaction of *L. taurica* and the biocontrol agents *Trichoderma harzianum* T39 (TRICHODEX) and *Ampelomyces quisqualis* (AQ10), alone or mixed with the mineral oils JMS Stylet-Oil and Add, respectively, were studied. The effect of the control agents on important life cycle stages of *L. taurica* (viability of conidia, conidia germination, leaf colonization by hyphae and disease severity on leaves) were studied under controlled conditions and in a greenhouse. In field experiments the above and other treatments were examined under various micro-climatic conditions. The biocontrol agents were more effective in disease control at 15 and 25°C than at 20°C, and there was less colonization of the leaf by the mycelium of the pathogen. The mineral oils and TRICHODEX reduced conidia germination. Under commercial greenhouse conditions, disease control by the various agents and by a treatment of alternation of the control agents were more efficient under warm conditions (above 25°C during the day – achieved by curtain closure – and 19°C at night) than at lower temperatures (20 and 13°C, respectively). A treatment of alternation of 'friendly' treatments (helio-sulfur, TRICHODEX, Neemgard and AQ10) in the warm greenhouse climate, resulted in effective disease control, similar to that by the standard alternation and mix of chemical agents. In colder greenhouses the former treatment was inferior to the chemical treatment. In the greenhouse the combination of high temperature with alternating sprays of the 'friendly' agents, controlled powdery mildew on pepper effectively.

Key words: Integrated disease management

Introduction

Leveillula taurica, the causal agent of powdery mildew is a widespread pathogen that causes severe damage in many crops. In pepper the symptoms may appear 50 days or more after planting; they may cover the entire lower side of the leaves and also the upper sides. Yellowing occurs on the upper parts of the leaves and necrotic lesions can frequently be seen. The most important damage in pepper is leaf shedding and exposure of the leaves to solar radiation. Thus, the quantity and quality of the fruits are harmed (Palti, 1988). Our research focused on the control of the disease by the use of climate management in the greenhouse and by application of the biocontrol agents TRICHODEX (*Trichoderma harzianum* T39) and AQ10 (*Ampelomyces quisqualis*) (Elad et al., 1998), the vegetable extract (Neemgaurd) and chemical fungicides. We report the results of experiments under controlled conditions and

under commercial greenhouse conditions in which integration of climate control with environment friendly agents were tested.

Materials and methods

Pepper (*Capsicum annum* L.) plants cv. Mazurka were planted in 2-L pots for the controlled conditions experiments and were infected at age 2 mo. Plants in a commercial greenhouse were planted in soil on 1 Sept. and were maintained as recommended for pepper growing in southern Israel. Control agents used were 1% Heliosulfur, 0.4% TRICHODEX (*Trichoderma harzianum* T39), 0.3% JMS Stylet-Oil, 0.03% AQ10 (*Ampelomyces quisqualis*), 0.3% AddQ oil, 1.0% Neemgaurd (neem oil) and 0.05% Amistar (azoxystrobin). Conidia of *Leveillula taurica* were collected dry from infected pepper plants; they were spread on leaves that had been treated with the control agents and observed under a light microscope 24 h later. In growth chambers (Conviron, Canada) with a 12-h light cycle plants were irrigated and fertilized as necessary. In all experiments disease severity was evaluated according the percentage of the leaf covered with disease symptoms. Results were presented as percentage coverage or as an area under disease progress curve (AUDPC), for which the disease severity curves were used as basic raw data.

Results and discussion

Climate management

Plants grown in a commercial greenhouse were subjected to two climate regimes: i. Conventional growth conditions for pepper, i.e., temperatures between 15 and 20°C during November to February; ii. temperature of 25±5°C. At the end of the experiment disease severity reached 92% leaf coverage in the 15-20°C treatment, whereas it reached only 34% severity in the 25±5°C treatment. It was concluded that a warm climate regime could be used for restraining epidemic outbreaks. Furthermore, it was suggested that application of control agents might result in further suppression of the disease, and several biocontrol agents were tested for this purpose.

Effect of control agents on Leveillula taurica

Conidia germination

Germination of *L. taurica* conidia was tested on pepper leaves that had been treated with each of the control agents (Table 1). The germination rates ranged between 50% in the untreated control and no germination in the sulfur-treated leaves. AQ10 did not affect germination whereas the oils AddQ and JMS Stylet Oil, and the biocontrol agent TRICHODEX reduced it by 48-86% (Table 1).

Table 1. Effect of control agents on germination of *Leveillula taurica* on pepper leaves at 20°C and 90% r.h.

Treat No.	1	2	3	4	5	6	7	8
	-	AQ 10	AddQ	2+3	TRICHODEX	JMS S-oil	4+5	Sulphur
Conc. (%):	0	0.03	0.3		0.4	0.3		1.0
% germination	50 a	47 a	21 bc	16 bc	26 b	7 cd	9 cd	0 d

Numbers followed by a common letter do not differ significantly from one another ($P \leq 0.05$).

Powdery mildew control under controlled climate conditions

In growth chambers disease severity was higher at 20 °C than at 15 and 25°C (Table 2). AQ10 controlled the disease significantly at 25°C and TRICHODEX did so at 20 and 25°C. Sulfur and the oils AddQ and JMS Stylet-Oil reduced the powdery mildew at all tested temperatures (15-25°C). The combination of the oils with the respective biocontrol agents was significantly similar to the effect of the oils alone (Table 2). The conclusion out of these experiments was that the additive effect of the biocontrol agents could be expected to yield beneficial results at the higher temperatures.

Table 2: Control of *Leveillula taurica* powdery mildew on leaves of pepper plants grown in growth chambers and incubated at three temperatures. Data of AUDPC are presented.

a. AQ10 and associated treatments

Treat No.:	1	2	3	4	8
	-	AQ 10	AddQ	2+3	Sulphur
Conc. (%):	0	0.03	0.3		1.0
15 °C	310 a	200 ab	110 bc	90 bc	10 c
20 °C	990 a	780 a	210 b	95 b	0 b
25 °C	320 a	5 b	10 b	10 b	0 b

b. TRICHODEX and associated treatments

Treat No.:	1	4	5	7
	-	TRICHODEX	JMS Stylet-oil	4+5
Conc. (%):	0	0.4	0.3	
15 °C	680 a	550 ab	320 bc	210 c
20 °C	3400 a	1550 b	550 c	690 c
25 °C	590 a	30 b	30 b	10 b

Numbers in each row followed by a common letter do not differ significantly from one another ($P \leq 0.05$).

Suppression of* Leveillula taurica *under commercial conditions

The biocontrol agents, the chemical fungicide Amistar, the neem extract Neemgaurd and sulphur were sprayed on pepper plants under commercial conditions, and disease severity was monitored for 2 months (Figure 1). Satisfactory disease suppression was obtained in the TRICHODEX + JMS, Amistar and Neemgaurd treatments. Excellent control was achieved with the sulphur sprays.

Integrated management of powdery integrated management of powdery mildew on pepper

In the last experiment we combined climate control with alternation of control agents. The conventional climatic regime (15-20°C) was compared with the higher temperature regime (25±5°C).

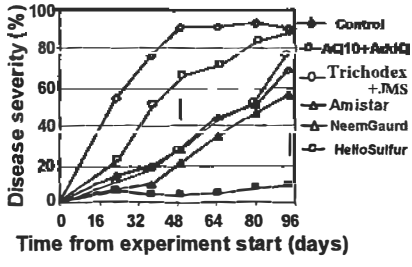

Figure 1. Control of *Leveillula taurica* powdery mildew on leaves of pepper plants grown in a commercial greenhouse. Agents were applied thirteen times on a weekly basis. Bars = LSD.

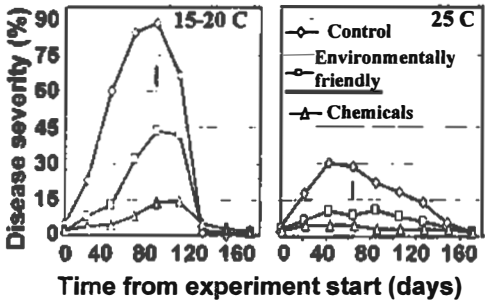

Figure 2. Control of *Leveillula taurica* powdery mildew on leaves of pepper plants grown in a commercial greenhouse. Agents were applied seven times on a weekly basis. Environmentally friendly = alternation of TRICHODEX, AQ10 and Neemgaurd. In the "Chemicals" treatment chemical fungicides were applied. Bars = LSD.

The combination of warmer climate with alternation of environment-friendly agents (TRICHODEX, AQ10 and Neemgaurd) resulted in satisfactorily low powdery mildew levels (Figure 2).

Acknowledgements

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Combined effect of microclimate and dose of application on the efficacy of biocontrol agents for the protection of pruning wounds on tomatoes against *Botrytis cinerea*

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Abstract: The efficacy of two fungi shown in earlier studies to provide high levels of protection on pruning wounds, *Ulocladium atrum* and *Microdochium dimerum*, was evaluated in precisely regulated growth chambers over a range of conditions known to occur in commercial greenhouses in southern France. The effect of microclimate was not identical for all components of biocontrol efficacy. It was most pronounced on the ability of the biocontrol agents (BCAs) to prevent or reduce the incidence of wound infection by *B. cinerea*. The efficacy of protection was mostly influenced by temperature rather than by relative humidity. Wound protection was reduced at 10°C and increased significantly with increasing temperatures for both BCAs. Other components of biocontrol efficacy, such as the ability to delay the outbreak of wound infection and the ability to slow down lesion expansion, were little influenced by climate. The observation of microclimatic effects on efficacy of biocontrol was highly influenced by the dose of BCA applied to the plants. Effects such as described above were strongly attenuated or completely masked when BCA spores outnumbered the pathogen's 10 to 1 after inoculation. In this situation, wound protection was uniformly high over most climate regimes, suggesting that doses may be adjusted to provide satisfactory levels of efficacy in conditions unfavourable for biocontrol. This study demonstrated that *U. atrum* and *M. dimerum* might be useful for wound protection of greenhouse tomatoes in a wide variety of microclimatic conditions, including those occurring in minimally heated structures. Those results concur with several years of trials at crop level. Combined with estimates of naturally occurring inoculum levels of *B. cinerea* and data on the dynamics of *B. cinerea* and the BCA in the wounds, these results should provide useful information to adapt inoculum doses to the environmental conditions for achievement of optimal disease control.

Key words: Botrytis, grey mould, tomato, biological control, microclimate, dose

Introduction

Infection of pruning wounds and stem wounds by *Botrytis cinerea* in protected tomato production are found in France both in minimally heated tunnels and in heated glasshouses (with sophisticated microclimatic regulation) where they constitute the most damaging Botrytis symptom. They can occur in a wide range of microclimatic situations, some of which may not be favourable to the efficacy of biocontrol agents (BCAs). The main purpose of the present study was to evaluate the effect of temperature and relative humidity on the efficacy of *Ulocladium atrum* and *Microdochium dimerum*, two fungi shown to provide high levels of protection on pruning wounds (Decognet *et al.*, 1999; Fruit & Nicot, 1999). It was further hypothesised that the dose of application of the BCAs might influence the impact of microclimate on efficacy.

Materials and methods

The same fungal isolates were used throughout this study: monoconidial tomato isolate BC1 of *B. cinerea* (Nicot et al., 1996), isolate Ua385 of *U. atrum* (kindly provided by J. Köhl) and isolate L13 of *M. dimerum* (obtained from a healthy pruning wound in a tomato greenhouse with particularly high incidence of grey mould). We used potted tomato plants cv Raissa and cv Felicia. The plants were produced in a heated greenhouse and used when they had 7-9 fully expanded leaves. The plants were watered daily with a nutrient solution. Watering was interrupted 8-24 hours before the plants were used in experiments to avoid guttation following leaf removal and to facilitate the absorption of inoculum into the wounds.

The effect of microclimate on efficacy of biocontrol was evaluated in multifactorial studies conducted in precisely regulated growth chambers (Boulard et al., 1996). Eighteen regimes were tested, combining a temperature of 10°C, 15°C, 20°C, 25°C, 30°C or 35°C with a relative humidity (RH) of 50%, 70% or 90%. Separate tests with different inoculum concentrations and methods of inoculation were designed to evaluate different components of biocontrol efficacy. For each climate regime, three critical steps of the disease cycle were considered: (1) infection of pruning wounds; (2) lesion expansion and (3) sporulation on diseased tissue. Five plants were used for each climate regime and each type of wound treatment under study and the whole set of experiments was repeated at least 3 times in 1998-2001 for each BCA (up to 5 times). Pruning wounds were inoculated with *B. cinerea* alone or together with either BCA. The number of infected pruning wounds per plant and if applicable, the length (in mm) of lesions that developed on the petiole stubs and on the stems were recorded daily between the third and seventh days after inoculation. Spore production was quantified on stem lesions seven days after inoculation in all tests with *U. atrum*.

To evaluate the combined effect of climate and dose of BCA application, varying inoculum concentrations were used in some of the replicated studies, to obtain different BCA-pathogen ratios in the initial microbial populations.

Results

Effect of microclimate on efficacy of wound protection

Infection of pruning wounds on plants inoculated with *B. cinerea* alone was observed in all conditions tested except those with a temperature of 35°C. The highest rates of disease incidence were recorded at 90% relative humidity and the lowest at 50% (Figure 1). They tended to be lower at higher temperatures.

Treatment of wounds with either *U. atrum* or *M. dimerum* immediately after inoculation with *B. cinerea* significantly ($P<0.01$) reduced the incidence of wound infection (Figure 1). However, the effect of the biocontrol agents was not identical over the different climate regimes. Infection levels on BCA-treated plants were distinctly lower at higher temperatures and also somewhat lower at 90% and 70% RH than at 50%.

To distinghuish the effect of microclimate on biocontrol from that on the infection process by *B. cinerea* alone, a protection index was computed as:

$$\text{Protection Index} = 100 \times (\text{Disease}_{\text{control}} - \text{Disease}_{\text{BCA}}) / \text{Disease}_{\text{control}};$$

where "Disease" represented the area under the progress curve over 7 days after inoculation for plants inoculated with *B. cinerea* alone ("control") or together with a BCA ("BCA"). Analysis of variance on these data (Figures 2 A and C) indicated a significant effect of

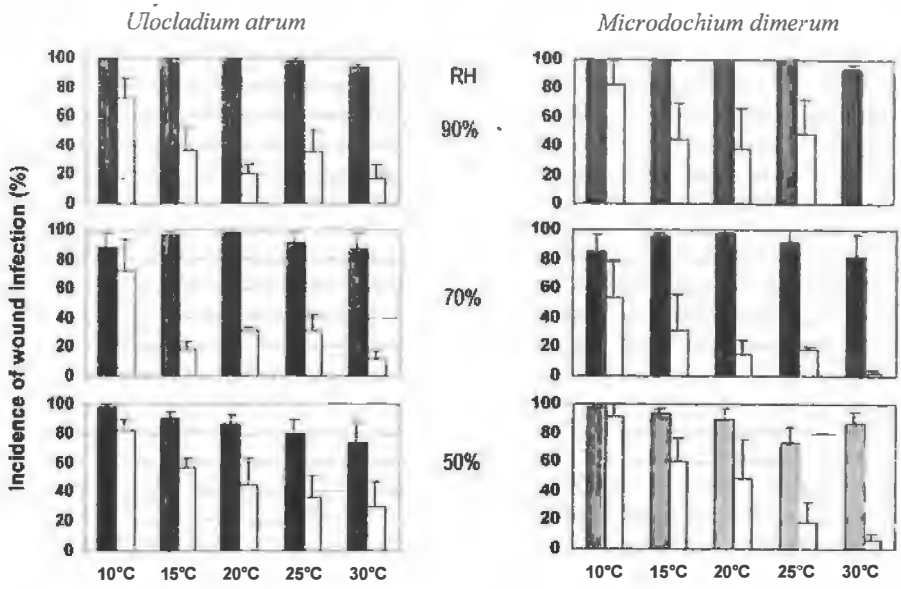

Figure 1. Effect of temperature (°C) and relative humidity (RH) on the incidence of wound infection 7 days after inoculation with *Botrytis cinerea* alone (■) or together with a biocontrol agent (□). Both the pathogen and the biocontrol agents (*U. atrum*, left and *M. dimerum*, right) were inoculated at 10^7 spores/m.l. Error bars indicate the standard errors of the means.

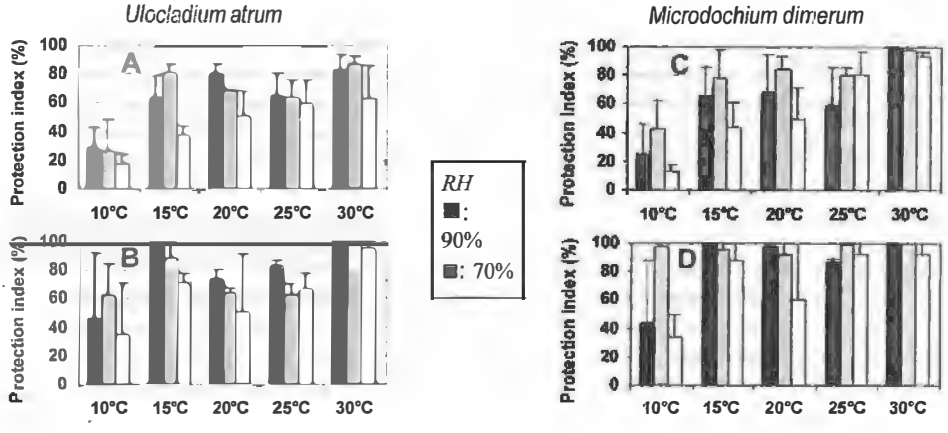

Figure 2. Effect of temperature (°C) and relative humidity (RH) on protection of tomato pruning wounds by *U. atrum* (left) and *M. dimerum*, right (applied at 10^7 spores/ml) against infection by *Botrytis cinerea*. Protection indices were computed on the basis of percent infected petiole stubs 7 days after inoculation with *B. cinerea* at 10^7 (A, C) and 10^6 (B, D) spores/ml. Error bars indicate the standard errors of the means.

temperature but not of relative humidity (for example, p values of 0.001, 0.17 and 0.81 for T, RH and T*RH interaction factors, respectively, for *M. dimerum*). Efficacy of both BCA's was distinctly lower at 10°C and increased with increasing temperatures.

Effect of microclimate on other components of biocontrol efficacy

In cases when wound infection was not prevented by treatment with the BCAs, their ability to delay the outbreak of lesion development was monitored. Both BCAs had a significant effect (average delay of 0.7 day; $P<0.001$ for *M. dimerum* for example), but a high level of variability was recorded over the different climate regimes and no significant temperature or RH effects were observed (for example, $P>0.21$ and 0.38, respectively, for *M. dimerum*). Similarly, the ability to slow down lesion expansion and to inhibit sporulation of *B. cinerea* was variable and little influenced by climate.

Combined effect of dose and microclimate on efficacy of biocontrol

When the dose of BCA applied to the wounds was ten times that of the inoculum of *B. cinerea,* the protective effects of *U. atrum* and *M. dimerum* were strongly increased (Figures 2 B and D) and temperature no longer influenced significantly their efficacy ($P>0.08$ for *M. dimerum*, for example). Other components of efficacy were also significantly strengthened in the rare cases when lesions developed (eg, average delay of 1.2 days in the outbreak of lesions and reduction of lesion expansion from 6.6 mm/day for control plants to 3.3 with *M. dimerum*). No sporulation of *B. cinerea* was observed on any of the wounds treated with the "high" dose of *U. atrum*.

Discussion

This study demonstrated that *U. atrum* and *M. dimerum* may be useful for wound protection of greenhouse tomatoes in a wide variety of microclimatic conditions. Those results concur with several years of trials at crop level. The generally limited effect of microclimate (particularly that of relative humidity) on efficacy of wound protection merits further attention, as both temperature and relative humidity are known to exert a high influence on the epidemiology of diseases caused by *B. cinerea*. Combined with estimates of naturally occurring inoculum levels of *B. cinerea* and data on the dynamics of *B. cinerea* and the BCA in the wounds, these results should provide useful information to adapt inoculum doses to the environmental conditions for achievement of optimal disease control.

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Ecophysiological manipulation of solid and liquid fermentation affects yield and viability of the biocontrol agent *Metarhizium anisopliae*

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Abstract: The fungal biocontrol agent *Metarhizium anisopliae* was cultivated in solid and liquid fermentation under stress free and water stress conditions. Conidial production decreased with increasing stress but conidial viability increased. In liquid culture, blastospore production increased under water stress conditions compared to stress free conditions. As water stress increased, endogenous erythritol content of blastospores increased. Erythritol can act as a compatible solute reducing the intracellular water potential and enabling faster germination at a wider range of environmental relative humidity. When harvested blastospores were washed with isotonic solution equivalent to the growth medium retained higher endogenous mannitol and erythritol content compared to water-washed blastospores, while at the same time retaining higher viability.

Key words: Conidia, blastospores, polyols, water activity

Introduction

One of the most crucial factors that limits the success of a fungal biocontrol agent (BCA) is environmental relative humidity (ERH). Infective fungal propagules can be rendered ineffective at sub-optimal water availability (Hallsworth & Magan, 1995). Therefore, methods for producing fungal propagules should aim not only at high spore yield but also at improving inoculum quality. Fungi have no mechanisms for the active uptake of water, instead the driving force for water inflow is the difference in water potential between the fungal cell and the external environment (Jennings, 1995). Ability to germinate under low water availability will be therefore greatly influenced by the intracellular water potential in relation to the external water availability. Fungal cells have been found to accumulate compatible solutes in response to water stress, particularly polyhydric sugar alcohols (polyols), reducing the intracellular water potential (Jennings, 1995). Thus, by reducing the intracellular water potential, polyols facilitate water uptake by osmosis. Manipulation of the polyol content of fungal cells by culturing fungi under water stress condition can lead to the production of propagules of improved viability even at low environmental RH. In this paper we present work on the effect of environmental manipulation of solid and liquid fermentation, through water stress conditions, on the production, viability and polyol content of spores of the BCA *Metharizium anisopliae*.

Materials and methods

Organisms and media

M. anisopliae (416.96) (Horticulture Research International (HRI), Wellsbourne) was grown on malt extract agar (Merck, U.K.) at 25°C for 10-14 days before it was used in any of the experiments. Solid substrate medium used in this study was a wheat-based product. It was modified to saturation (0.99 $a_w \equiv$ 99% E.R.H.) and to 0.98 and 0.96 a_w by addition of water.

Glycerol/water solutions were also used to modify the substrate to these same treatments. Modified grain treatments were placed in 1 l Duran bottles and equilibrated for 2 days at 4° C.

We used Adàmek's (1963) medium, except that cornsteep solid was used instead of cornsteep liquor. The medium a_w was 0.99. For a_w modification, KCl (2.24 %; 0.98 a_w, or 3.73 %; 0.97 a_w) or NaCl (1.5 %; 0.98 a_w, or 2.9 %; 0.97 a_w) was added to distilled water and after measurement of final volumes the medium was prepared again (Adàmek, 1963).

Inoculation, harvesting procedure and germination tests

A subsample of 20 g of modified solid medium from each treatment was placed in vented jars and autoclaved for 50 min at 121°C on two consecutive days and then inoculated with 0.25 ml inoculum of $5x10^6$ spores/ml. Jars containing modified grain of the same a_w were placed together in closed sandwich boxes where environmental ERH was maintained at the treatment ERH using glycerol/water solutions and incubated at 25°C. Spores were harvested by washing the substrate in sterile distilled water + 0.01% Tween 80. The resultant liquid was filtered through one layer of Lens cleaning tissue (Whatman 105), and a subsample counted using a haemocytometer. The filtrate was then centrifuged at 3000 rpm for 10 min and spores were used for viability studies. The rest was frozen for sugar alcohol analysis using HPLC. Baffled flasks (250ml Nalgene) containing 50 ml of liquid medium were sterilised (15 min, 120°C) and inoculated with 0.5 ml of $5x10^6$ spores/ml. Inoculated flasks were incubated at 25°C on a rotary shaker (180 rpm). For germination and polyol studies, blastospores were harvested (filtration through Lens cleaning tissue) when production reached its maximum. After centrifugation of the filtrate, the resultant spore pellet was re-suspended in 10 ml of sterile water and re-centrifuged. Spores from the pellet were used for viability tests and the rest was kept frozen for HPLC analysis. The experiment was repeated using PEG 200/water solution equivalent to the culture medium a_w, instead of water for washing the blastospores.

In the case of solid substrate fermentation conidia from the pellet formed as described above were suspended in sterile water/0.01%(w/v) Tween 80 solutions to give spore suspensions of approximately 10^6 to $5x10^6$ spores/ml. Germination was checked on water-stressed media (300g of PEG 200/l (Sigma, U.K.) + 0.1% malt extract and 2% technical agar No 3 of the final volume). The resultant a_w levels of the medium was 0.963. A 0.1 ml of spore inoculum was used and the incubation time was 34 h.

Extraction and detection of polyols and sugars

The procedure followed has been described in detail previously (Hallsworth & Magan, 1995)

Results

Production, viability and polyol content of conidia

For either 5 or 10-day old cultures conidial production was highest at high a_w than at lower a_w. Specifically for 5-day-old cultures conidial production was as follows: $6.19x10^7$ (saturated: 0.99 a_w), $3.83x10^7$ (0.98 a_w), $3.09x10^7$ (0.98 a_w modified with glycerol/water solution), $6.63x10^6$ (0.96 a_w), $1.3x10^7$ (0.96 a_w modified with glycerol/water solution) spores/g fresh grain weight. For 10-day-old cultures conidial production was: $6.25x10^8$ (saturated: 0.99 a_w), $4.27x10^8$ (0.98 a_w), $3.48x10^7$ (0.98 a_w modified with glycerol/water solution), $1.26x10^8$ (0.96 a_w), $2.83x10^7$ (0.96 a_w modified with glycerol/water solution) spores/g fresh grain weight.

Overall, the lower the a_w of the production medium the higher the viability of the produced conidia expressed either as percentage germination or germ tube length. For all treatments, conidial viability decreased with culture age. Polyol analysis of conidia did not show any marked differences between treatments (data not shown).

Production, viability and polyol content of blastospores

A significant increase in blastospore production occurred when *M. anisopliae* was grown in water-stressed Adàmek's liquid medium compared to unmodified medium (0.99 a_w) (Figure 1). The peak of blastospore production was dependent on the a_w of the medium and it occurred at 72 h for the unmodified and the modified to 0.98 a_w and at 86 h for the modified to 0.97 a_w. The viability of blastospores washed with water or isotonic PEG 200 solution (expressed either as percentage germination or germ tube length) from different treatments is shown in Figure 2. Viability of blastospores washed with isotonic solution was clearly improved when compared to water-washed blastospores.

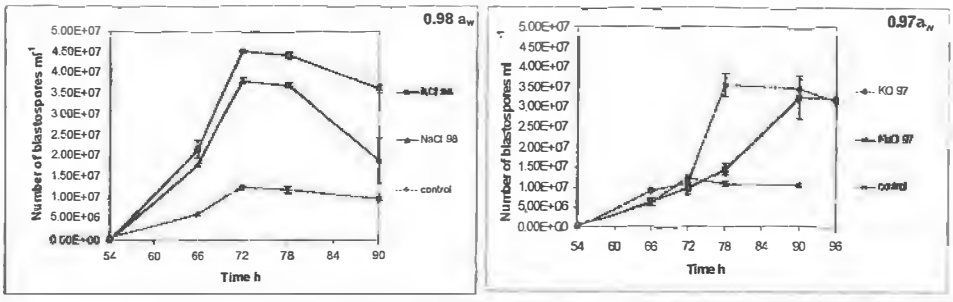

Figure 1. Temporal blastospore production in unmodified/modified Adàmek's medium. The medium was modified to 0.98 and 0.97 a_w with either KCl or NaCl. Blastospore production in the (unmodified) medium (0.99 a_w) is presented in both figures. Bars represent SED, (n=3).

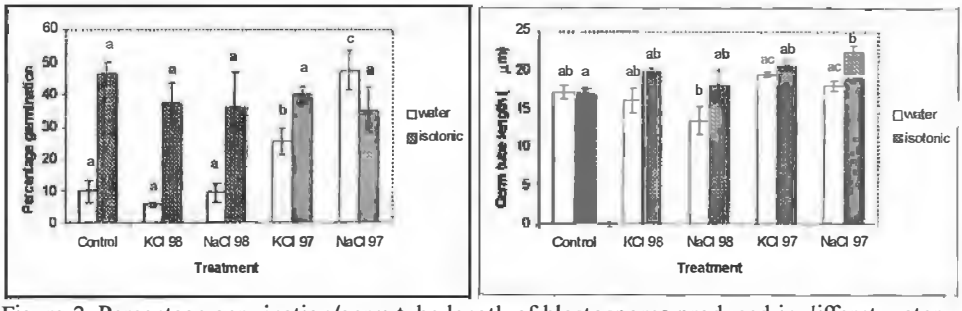

Figure 2. Percentage germination/germ tube length of blastospores produced in differnt water stress conditions. Blastospores from the unmodified and the modified to 0.98 a_w were harvested after 72 h incubation and from the modified to 0.97 a_w after 86 h incubation. Bars= SED, (n=3). Different letters for each washing solution indicate significant treatment differences ($P \leq 0.05$).

Discussion

On solid substrate fermentation spore production was decreased with increasing water stress but spore viability increased. The improvement in viability with increased water stress was strongly and negatively correlated (pearson coefficient – 0.75; data not shown) to the number of spores produced. It could be possible that when fewer spores are produced they are provided with a more optimum nutritional environment affecting the accumulation of

endogenous reserves. Increased water stress in liquid fermentation increased blastospore production, possibly through the effect of a_w on the morphology and the growth of the fungus. The lower molecular polyol erythritol increased in blastospores produced under water stress conditions. Low molecular polyols are more effective in reducing intracellular water potential enabling faster water inflow and subsequent germination (Hallsworth & Magan, 1995). When blastopores were washed with isotonic solutions they contained remarkably more erythritol and mannitol compared to water-washed blastospores. Accordingly, blastospores washed with isotonic solutions showed improved germinability compared to water-washed blastospores. The fact that conidia from the solid substrate experiment were washed with water could explain why no significant polyol accumulation was detected. Therefore, harvest and post-harvest treatment of spores could have major implications for the development of a biologically competent formulation. Potential exists in exploiting such conditions for improvement of inocula mass production of fungal BCAs with improved field performance.

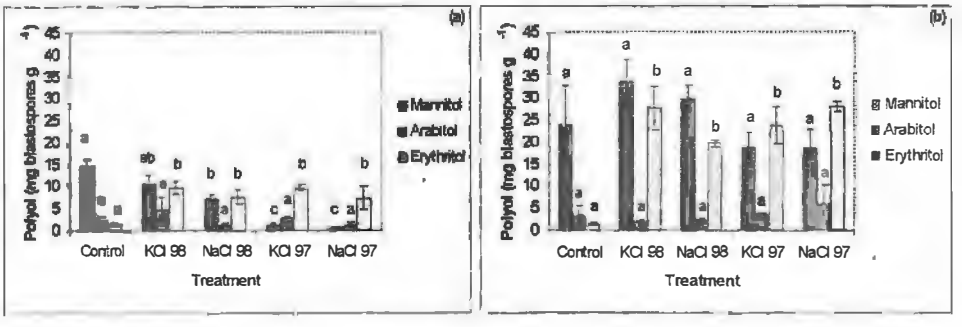

Figure 3. Water stress effect on polyol pattern in blastospores of *M. anisopliae*. Blastospores were washed with (a) water or (b) with isotonic solution. Bars represent SED, (n=3). Different letters for each polyol indicate significant treatment differences ($P < 0.05$).

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Soil factors and biocontrol

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The effect of soil chemical composition on the biocontrol of *Rosellinia* in cocoa

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Abstract: We investigated the effect of soil pH, organic matter and phosphate on control efficacy of *Rosellinia* root rot in cocoa by biocontrol agents applied as single strains or in mixtures. *In vitro* growth rates were influenced by pH and phosphate. Seedling bioassays showed that pH was the most important factor followed by organic matter. Phosphate content was the least important factor. Consistent control behavior was obtained only by the biocontrol mixtures. Recommendations are given for control of *Rosellinia* root rot in cocoa.

Key Words: biocontrol, cocoa, organic matter, pH, phosphate, *Rosellinia, Theobroma cacao*

Introduction

Rosellinia root rot of cocoa (*Theobroma cacao*) is an important soil-borne disease in Latin America and the Caribbean causing annual tree mortality of up to 7% (Cadavid, 1995). The causal agents are *Rosellinia pepo* and *R. bunodes*. The disease is mostly associated with fairly acidic soils, rich in organic matter (OM) with a high humidity level (López & Fernández, 1966). Furthermore, it has been suggested that the disease is favored by soils with low phosphate content (Waterston, 1941). The objective of this study was to determine the effect of soil pH, OM and phosphate level on biocontrol of *Rosellinia* in cocoa using single strains or mixture of strains of the antagonistic fungi, *Clonostachys* spp. and *Trichoderma harzianum*. Both genera also prefer acidic soils with a high OM content.

Material and methods

Effect of pH and phosphate on *Rosellinia* isolates (RB1, RB2, RB3 and RB4) and selected mycoparasites were determined *in vitro*. Potato dextrose agar was adjusted to different pH levels (range 3-10). The effect of phosphate (range 0.00-0.10M, 0-9500 ppm) was measured with two different phosphate sources: K_2HPO_4 and NaH_2PO_4. Based on prescreens, isolates of *Clonostachys* spp. (ARB11 and ARB37) and *T. harzianum* (ARB4 and T-22) were used in seedling bioassays in natural soils. In these tests, effects of soil pH (range 4-6), phosphate concentration (range 0-1000 ppm) and OM content (range 6-12%) on biocontrol efficacy, were determined. *Rosellinia* was applied at a dose of 250 g colonized wheat seeds per 2 kg of seedling bag. The antagonists were applied simultaneously either singly or as a mixture (0.5 l of $1x10^6$ spores/ml). Soil pH was adjusted using $CaCO_3$, OM levels using bokashi (Sasaki, 1994), phosphate, using NaH_2PO_4. There were five replicates per treatment. Plants were

checked weekly and disease severity scored according to a non-parametric scale. In addition, the following parameters were measured: days of survival, plant height, dry weight of roots, root length, dry weight of leaves, leaf area, number of leaves and percentage mortality. Treatment means were compared with analysis of variance with the appropriate data transformation when the distribution needed to be normalized.

Results

In vitro growth rates of all fungi were influenced by pH with the optima usually coinciding with the pH of the soil of origin (Table 1).

Table 1: Optimum pH levels for *in vitro* growth. In parentheses pH of the soil of origin

Rosellinia sp	Optimum pH	Mycoparasites	Optimum pH
RB1	4 (4.7)	ARB4	4 (4.7)
RB2	6-8 (5.5)	ARB11	≥ 5 (5.5)
RB3	4-6 (4.7)	ARB37	6-7 (4.7)
RB4	4-5 (4.7)	T-22	6 (n.k.*)

*n.k. = not known

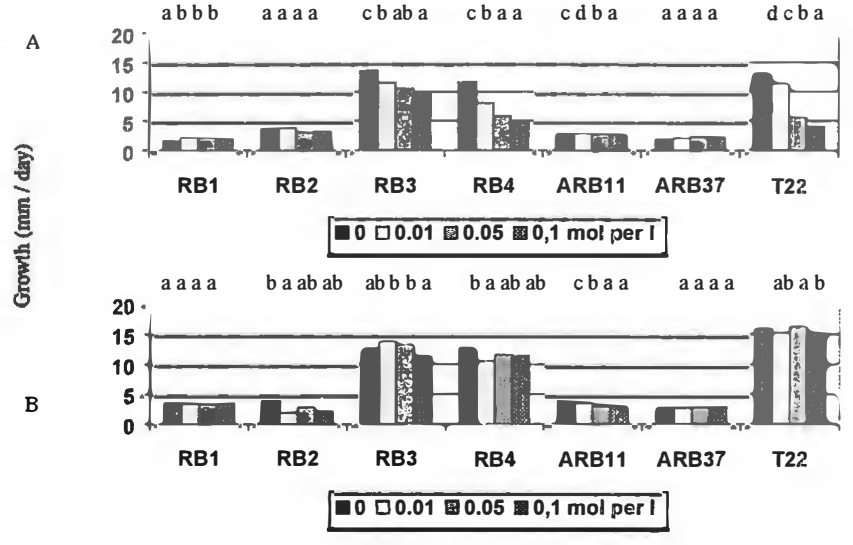

Figure 1. Effects of phosphate on four *Rosellinia* isolates (RB1, RB2, RB3, RB4) and mycoparasites *Clonostachys rhizophaga* (ARB11), *C.* cf. *byssicola* (ARB37) and *Trichoderma harzianum* (T-22). a) K_2HPO_4 and b) NaH_2PO_4.

The effect of phosphate was dependant on the phosphate source and could not be explained by concomitant changes in pH-values alone (Figure1). For all variables in all three bioassays, we found significant differences between the treatments. Five out of the nine plant growth parameters were influenced by soil pH rendering biocontrol more efficient at high pH levels. A mixture of three antagonists suppressed disease severity significantly ($P \leq 0.001$) better than a mixture of two antagonists, but this effect was dependent on the pH (Figure 2a). OM

was the second most important factor with two parameters being affected. Although both the pathogen and its antagonists are favored by OM, high OM content invariably reduced biocontrol efficacy (Figure 2b). Phosphate additions influenced five of the nine plant growth parameters but did only affect the efficacy of biocontrol treatments with respect to root length. Phosphate levels were the least relevant factor within the realistic range for cocoa growth (Figure 2c). Strain mixtures performed consistently better than single strains and were less variable in their control efficacy.

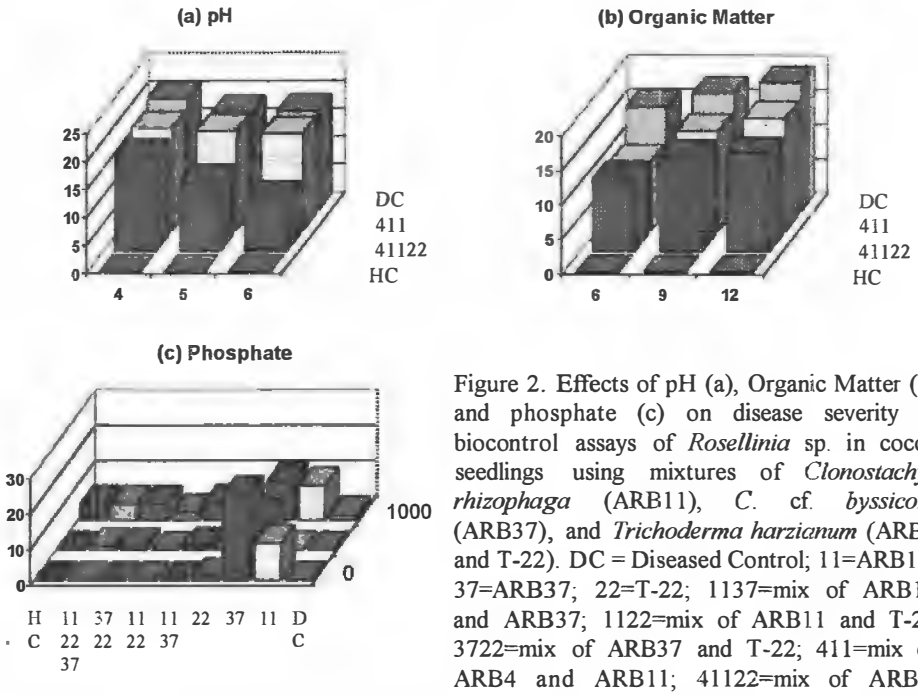

Figure 2. Effects of pH (a), Organic Matter (b) and phosphate (c) on disease severity in biocontrol assays of *Rosellinia* sp. in cocoa seedlings using mixtures of *Clonostachys rhizophaga* (ARB11), *C.* cf. *byssicola* (ARB37), and *Trichoderma harzianum* (ARB4 and T-22). DC = Diseased Control; 11=ARB11, 37=ARB37; 22=T-22; 1137=mix of ARB11 and ARB37; 1122=mix of ARB11 and T-22 3722=mix of ARB37 and T-22; 411=mix of ARB4 and ARB11; 41122=mix of ARB4, ARB11 and T-22; 112237=mix of ARB11, ARB37 and T-22; HC=Healthy Control.

Discussion

Organic matter amendments are used in the management of a wide range of soil-borne diseases (Papavizas & Lumsden 1980; Raguchander et al., 1998). Analyzing the effects of OM content on the effectiveness of biocontrol showed a clear tendency: when OM increased, mortality and disease severity also increased. We therefore conclude that *Rosellinia* is capable of exploiting these resources better than the biocontrol agents. The effect of soil pH on *Rosellinia* root rot is less conclusive than the effect of OM (Cadavid, 1995). In our study, however, pH influenced five of the nine growth parameters assessed, whereas OM influenced only two. We therefore conclude that pH is at least as important as OM. Although phosphate influenced five out of the nine plant growth factors, it did not affect biocontrol efficacy except for root length. However,

we did not find a simple relationship between phosphate levels and biocontrol efficacy. In our study, a mixture of three mycoparasites was less affected by changing pH and (to a lesser extent) OM values than a mixture of two mycoparasites. The three mycoparasites mixture performed consistently better and was more adaptable to variable soil chemical compositions. In the phosphate assay the mixture of three mycoparasites never differed from the healthy control and was significantly better than the diseased control for three out of the nine measured plant growth parameters. We recommend liming and the removal of coarse woody organic material in combination with antagonistic strain mixtures for optimizing biocontrol efficacy of *Rosellinia* sp. in cocoa.

Acknowledgements

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Soil structure, fungal exploration and consequences for biocontrol

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Abstract: Mycelial exploration in soil occurs within a heterogeneous medium through a network of pores, cracks and aggregates as fungi spread from a source of inoculum to a susceptible host. However, understanding mycelial spread through soil has long been limited by difficulties in observation and quantification of fungi as they spread through this matrix. In this paper we describe how population dynamics of fungi in soil can be quantified by linking experimentation in soil physics (to quantify and control soil structure) and soil microbiology (to visualise and quantify hyphae within soil) with epidemiology (to link mycelial growth with epidemics). The combination of these techniques, analyses and epidemiological models opens up new possibilities to understand the dynamics of disease in soil.

Introduction

Understanding the spatial and temporal dynamics of microbes in a complex heterogeneous and competitive environment is a key issue in soil microbiology. These dictate the outcome of many ecologically and economically important processes in soil, such as organic matter dynamics, remediation of contaminated soil, invasion of beneficial organisms or the spread of an epidemic. Soil structure provides the environment in which biological, physical and chemical interactions occur, and through which microbes interact and exploit resources in soil. Using carefully controlled experimental systems, novel microbiological techniques and mathematical concepts in epidemiology we can enhance our understanding of how soil physical conditions can limit invasion of soil-borne fungal pathogens, addressing fundamental questions ranging from exploration of soil by fungi at the microscopic scale to consequences for infection and epidemics. In this paper we discuss two examples. We show how the epidemiological concept of the pathozone is a convenient way to summarize the complicated dynamics of *Rhizoctonia solani* spreading through soil (Otten et al., 2001) and how novel thin sectioning techniques can be used to reveal the interactions between soil structure and fungal exploration that dictate the pathozone dynamics.

Pathozone dynamics as a link between soil physics and fungal epidemiology

One important feature in the invasion and persistence of many fungal parasites and saprotrophs in soil is the ability of fungi to spread by mycelial growth and associated expansion of fungal colonies. The role of soil structure in controlling the spread of fungal pathogens has received relatively little attention. We used a combination of epidemiological and soil-physical techniques to show that soil structure has a major impact on the ability of fungi to colonise

soil. We based this on quantification of the colonization efficiency for the soil-borne fungal plant pathogen *Rhizoctonia solani* spreading through replicated soil samples. The colonization efficiency is derived from the epidemiological concept of the pathozone (Gilligan, 1985; Gilligan & Bailey, 1997) and is quantified as the relationship between the probability of successful colonization of a target (in this case a sterilized quinoa seed) and distance from the source of inoculum (Bailey et al., 2000). The dynamics of colonization efficiency are quantified for the fungus spreading through a non-sterile sandy loam packed to obtain samples with different structures. Starting with identical air-filled pore volume, we used aggregate sizes and bulk-density to alter the geometry of the pore volume. The effect of bulk-density and aggregate-size on the air-filled pore volume was assessed by a combination of thin sectioning and pore-size distributions estimated from water retention characteristics. An increase in bulk-density and a reduction in aggregate-size increased the fraction of micropores in the air-filled pore volume at the expense of larger pores. These changes resulted in smaller, more slowly expanding fungal colonies (Figure 1). This work combined techniques in soil physics that control and quantify soil structure with epidemiological concepts to show that the spatial arrangement of pore-pathways in soil, as manipulated by aggregate-size, bulk-density, and water content have major consequences for the dynamics of fungal spread in soil. Quantification of fungal spread from particulate soil inoculum is an important step towards linking short-scale soil physical conditions with disease dynamics at larger scales.

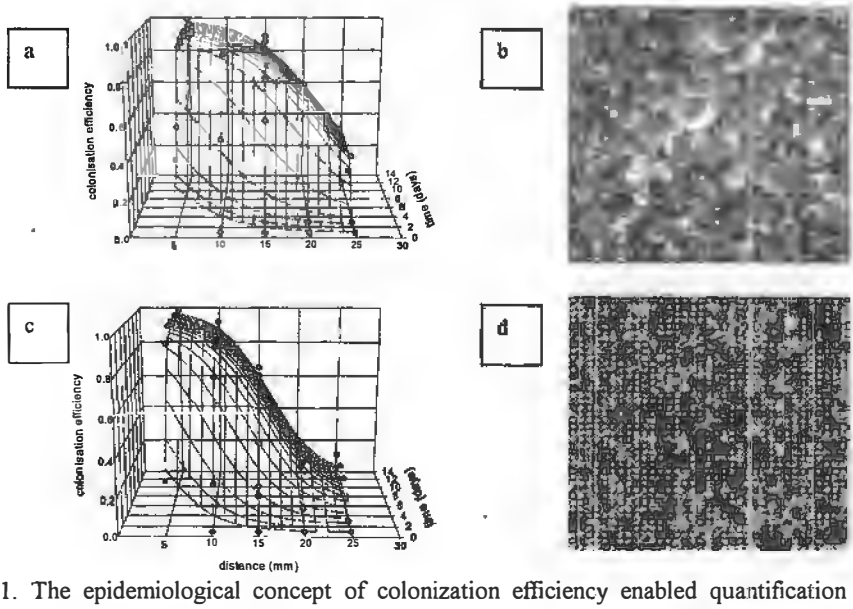

Figure 1. The epidemiological concept of colonization efficiency enabled quantification of fungal growth dynamics through non-sterile soil (a,c). Soil structure has a major impact on fungal spread. Smaller fungal colonies with a higher biomass density were formed in soil compacted at 1.5 Mg/m^3 (c,d) than at 1.3 Mg/m^3 (a,b). Soil structure is represented by porosity classes ranging from > 95% pore space (white) to < 5% pore space (black). The dots refer to the locations where fungal hyphae were detected (b,d). (Soil sections: 27 × 23 mm)

Soil thin sections reveal the spatial organization of fungi in soil

The pathozone dynamics are a convenient way to summarise complicated dynamics of fungal growth through soil, but it is also a black-box that hides the dynamics of hyphal growth and the local interactions with soil structure and competitors that lead to colony behaviour. Recent developments in thin sectioning technique (Harris et al., 2002) enabled quantification of the spatial organization of fungal hyphae *in situ*. First, we show how maps can be produced to quantify the distribution of hyphae within a fungal colony in soil. Then, we show how the technique can be used to relate this distribution to soil heterogeneity.

Production of hyphal distribution maps

We investigated the interaction between fungal spread and soil structure in replicated microcosms, using aggregate-sizes, bulk-density and air-filled pore volume as experimental variables to manipulate soil structure. The pathogenic fungus *R. solani* was locally introduced in replicated microcosms and allowed to spread for 5 days. Subsequently, large-scale biological thin sections were produced to obtain high resolution mapping of mycelial distribution in soil. The sections were 27 × 23 mm each, approximately equivalent to the size of a fungal colony initiated from a local nutrient source. To visualize fungal hyphae in soil-thin sections, observations at high resolutions are required. Therefore, we quantified fungal densities in microscopic images (0.44 mm^2) at high magnifications. At this scale, hyphae of *R. solani* were clearly visible under fluorescent light as fragments of fungal hyphae in the soil matrix. Typically, hyphae were shown to be present in void spaces in-between more dense solid mass, occupying less then 1% of the surface area within each microscopic view. There was no difference in hyphal density at this scale amongst sections that contained hyphae. In order to allow for quantification of hyphal distribution at larger scales and to provide a mechanistic link with the pathozone, we simplified the quantitative analysis to presence or absence of fungal hyphae in the microscopic sections. The mycelial distribution within the entire section (6.2 cm^2, equivalent to the size of a fungal colony) was subsequently quantified as the proportion of sites occupied by the fungus as a qualitative measure of hyphal density in different soil samples. This is the first time that information has been obtained on spatial distribution of fungal hyphae in soil at such a large scale whilst keeping the high resolution required for biological studies.

Soil heterogeneity and fungal exploration

We used the maps with the spatial distribution of mycelium in soil to investigate the relationships between small scale heterogeneity in soil and fungal exploration. First, pore maps had to be produced at the same scale and resolution as the maps for the spatial distribution of fungal hyphae. The same sections in which the fungal distribution was quantified (27 × 23 mm) were imaged again in one pass, i.e. at a lower magnification, and thresholded to produce binary pore maps. In order to match the spatial resolution of the mycelial maps (i.e., 0.77 × 0.58 mm), the pore maps were then deresolved by dividing the images into 1400 contiguous quadrats, and calculating the mean pixel value to within 1%: this equates to the average porosity of each quadrat, hereafter referred to as porosity class. The maps with spatial organisation of fungal hyphae could now be correlated with the underlying soil structure, represented by a porosity value. Thin sections were taken from replicated microcosms in which soil structure was manipulated by bulk-density. The hyphal distribution maps were combined with the deresolved pore maps of the same section. The volume of soil colonised by the fungus, quantified as the proportion of sites occupied by the fungus, increased with increasing bulk density of the soil. These results are consistent with reported data from literature and with previous work which showed that small fungal colonies with high biomass density are formed when the spread of a fungus is restricted by blockages of pores. Changes of fungal morphology with nutrient

distribution in the environment have frequently been studied, but this is the first time that evidence is provided that soil structure has an equally significant effect on fungal morphology.

The spatial distribution of mycelium within each fungal colony was patchier for fungi spreading through soil at lower bulk densities. To quantify the correlation between small-scale heterogeneity and fungal presence we calculated the proportion of microscopic sections in each porosity class that contained hyphae. Analysis revealed that: (i) fungal hyphae were present in all porosity classes, including the areas that contained < 5% visible pore space, and (ii) the fungus was biased towards areas within each soil sample that are higher in porosity and contain larger pores. This was particularly the case with a lower soil bulk density.

Ecological and epidemiological significance

Manipulation of soil structure is one of the principal means by which fungal and microbial dynamics can be controlled at the field scale. We identified soil structure as a major component governing the spatial exploration of soil by fungi. It is the distribution pattern at the scale of the microorganisms that will dictate the pathogen-antagonist encounters necessary for biocontrol. A classical example where unevenness of microbial function has long been recognized is that of denitrification. The effect of soil structure on the spatial distribution of fungi in soil is, however, also an important reality for epidemics and biocontrol. Faster movement of pathogenic fungi through soil along preferential pathways will not only speed up epidemics but also might enable fungal pathogens to outrun biocontrol agents. In particular, the presence of larger 'gaps' in the soil can be expected to provide highways for fungal spread, whilst establishing barriers for bacterial control agents. The effect of soil heterogeneity on interactions between organisms is however largely unknown. We believe that the new techniques, analyses and epidemiological models presented here have opened up new possibilities to understand the hitherto opaque dynamics of disease in soil.

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Soil moisture and root zone pH as tools for enhancing biocontrol of *Pythium* by *Trichoderma*

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Abstract: Soil moisture effects were determined using 36 independent hydroponic units. It was shown that management of soil moisture within artificial growing media can aid in the control of *Pythium* induced reductions in yield. Lettuce plants receiving optimum irrigation levels showed no significant reduction in yield when inoculated with *Pythium*. However, stresses related to over- or under-watering resulted in *Pythium* inoculation causing significant yield losses. In both cases, *Trichoderma* overcame these negative effects and achieved significant levels of disease control, but was more effective under higher soil moisture levels. Growth stimulation responses were also seen to increase with increasing soil moisture. These results show that *Pythium* control is best achieved through the integration of *Trichoderma* and optimum soil moisture. However, where soil moisture is above or below optimum, *Trichoderma* serves to minimize the negative effects of *Pythium* infection, providing a buffering capacity against the effects of poor soil moisture management. Nutrient solution pH in hydroponic systems was effectively used to enhance biocontrol of *Pythium* by *Trichoderma*. *Trichoderma* shows a preference for more acid pH's while *Pythium* prefers a pH between 6.0 and 7.0. *In vitro* tests showed that *Trichoderma* achieved greater control of *Pythium* at pH 5.0, with no control at pH 8.0. In field trials, pathogenicity by *Pythium*, and hence yield losses, were greatest at pH 6.0 and 7.0 but there was no significant reduction in yield at pH 4.0. Conversely, biocontrol activity by *Trichoderma* was greatest at lower pH's, especially pH 5.0.

Key words: soil moisture, pH, biological control, *Trichoderma*

Introduction

For most soil fungi, disease severity increases with increasing soil moisture. This observation is supported by the work of Pieczarka & Abawi (1978) who studied the effects of soil moisture and temperature on root rot of snap beans caused by *Pythium ultimum*. Wakelin et al. (1999) found that *Trichoderma koningii* in sterile soil showed greatest saprophytic growth at 70% soil water holding capacity (WHC), with little saprophytic growth occurring below 20% WHC. However, no literature could be found in which the interactive effects of soil moisture on these two biotic responses (infection and biocontrol) were reported. Similarly, pH has a major effect on microbial activity. Sharma & Gupta (1999) found sporangial germination in *P. ultimum* to be pH dependent. Germination occurred over a pH range of 5.6 to 7.0, with maximum germination at 6.4. *Trichoderma* on the other hand, favours more acidic pH levels. Harman & Taylor (1988) noted that *Trichoderma* species grew well at pH's around 3.0. Jeong et al. (1997) used these facts to improve the rhizosphere competence of *T. harzianum* at a practical level. Mycelial growth of *P. ultimum* was seen to be strongly inhibited by *T. harzianum* under acidic conditions (pH 4.5 and 6.0) compared with soils at pH 7.0.

Materials and methods

Soil moisture trials

Soil moisture trials were conducted using a vertical system of stackable polystyrene pots (Verti-gro®). Thirty six stacks were constructed, each with its own reservoir, containing a submersible pump and aquarium heater. All pumps were linked to a programmable logic controller (PLC) allowing independent irrigation regime for each stack. The stacks each consisted of four pots, allowing for 16 plants per replicate. Optimum soil moisture and air filled porosity for lettuce production in this system using a 30% coir + 70% coarse potting mix medium was determined in preceding trials. Subsequent treatments were based on optimum, double and half irrigation. Treatments consisted of un-inoculated controls, inoculated controls (with *Pythium*), biocontrol treatments (*Pythium* + *Trichoderma*) and growth stimulation treatments (*Trichoderma* only). Each treatment was repeated under each irrigation regime and replicated three times. Lettuce seedlings were transplanted into the system at two weeks. *Trichoderma* was added at transplanting in the form of a conidial suspension containing approximately $5x10^7$ spores/gram, diluted to 1 g/l and applied as a drench at 50 ml/plant. *Pythium* spp. (previously determined as being pathogenic on lettuce) was bulked up on water agar and transferred to the root zone of inoculated plants, 48 hrs after transplanting, as 1 cm^2 blocks of agar. After five weeks, plants were harvested and the total wet weights for each stack were graphed and compared.

pH trials

Bell tests (Bell et al., 1982) were performed using half strength nutrient agar adjusted to pH 5.0, 6.0, 7.0, or 8.0. For each pH the Bell tests were replicated three times, with both *Pythium* and *Rhizoctonia* being tested against *Trichoderma* Kd. Tests were rated according to the scale established by Bell et al. (1982) and average results tabulated.

Tunnel trials were conducted using 36 mini hydroponic troughs. Each trough had its own reservoir allowing the recirculating solution of each to be set at a different pH. Four different pH's were used (4.0, 5.0, 6.0 and 7.0). Treatments were the same as for the soil moisture trials with the exception of growth stimulation treatments which were not used. The three treatments were repeated at each pH level and each was replicated 3 times. Lettuce seeds were planted in perlite, in Speedling®-24's and grown for 10 days in a seedling greenhouse. Trays were then transferred directly to the troughs. *Trichoderma* Kd was added at 0.5g/l to each reservoir and *Pythium* inoculations were done as before. The pH of the solution was checked daily and adjusted when necessary. Harvesting was done three weeks later and total shoot wet weight was recorded for each replicate. Average total shoot wet weight was calculated for each treatment and graphed.

Results and discussion

Soil moisture trials

The effects of soil moisture are clearly illustrated in Figure 1. Under optimum soil moisture conditions *Pythium* inoculation was seen as having little effect on lettuce yield. Under water stressed conditions however, (over- or under-watered) the reductions in yield were significant. These results partially support those of Pieczarka & Abawi (1978) who cited a number of authors in stating that high soil moisture is necessary for survival, spore germination and

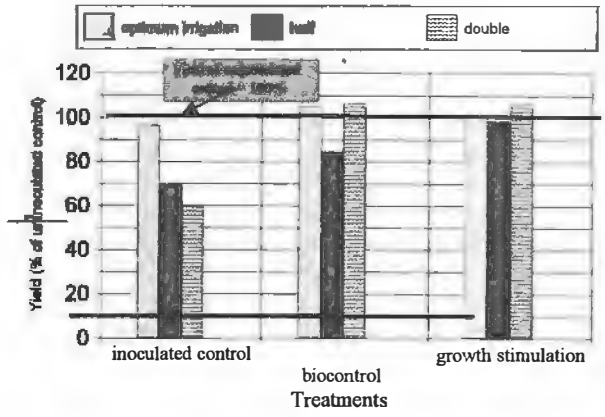

Figure 1. Effect of treatments on lettuce yield, expressed as % yield of uninoculated control

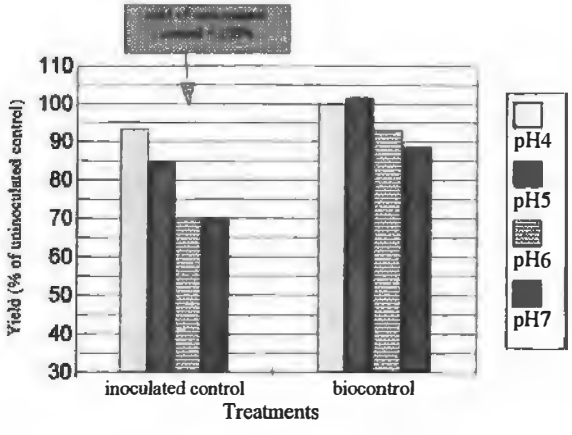

Figure 2. Effect of pH on treatments, expressed as % yield of un-inoculated control

Table 1. Effect of pH on Bell rating of *Trichoderma* Kd against *Pythium*

pH	Bell Rating	
	Rhizoctonia	*Pythium*
5.0	2.67	2.33
6.0	3.33	3.00
7.0	4.00	3.67
8.0	4.67	4.33

saprophytic growth of *Pythium* spp. and that wet soils provide an ecological advantage for *Pythium* spp., which are otherwise poor competitors. At low soil moisture levels the plants were probably sufficiently stressed to allow *Pythium* spp. to cause disease.

The low competitive ability of *Pythium* spp. in soil is highlighted by the fact that the addition of *Trichoderma* achieved significant biocontrol at all soil moisture levels. The results support the findings of Wakelin et al. (1999) in that *Trichoderma* performs better at higher soil moistures. This was evident in both the biocontrol and growth stimulation results, where higher yields were recorded in the over-watered treatments, followed by optimum watering. Under-watered treatments produced the lowest yields in terms of biocontrol and growth stimulation. Although it is difficult to apply this information in enhancing the efficacy of *Trichoderma* in the control of *Pythium*, it does show that where optimum soil moisture is difficult to obtain (e.g., high rainfall or high soil clay content) *Trichoderma* can provide a buffering capacity against the negative effects of poor soil moisture management.

pH trials

From Table 1 it can be seen that although all ratings were relatively low, biocontrol activity increased with decreasing pH. Field trial results as seen in Figure 2 show similar trends. Pathogenicity by *Pythium*, and hence yield losses, were greatest at pH 6.0 and 7.0. This supports the findings of Sharma & Gupta (1999). Conversely, biocontrol activity by *Trichoderma* was greatest at lower pH's, especially pH 5.0. This confirms the findings of Harman & Taylor (1988). On the basis of these results it was proposed that pH manipulation could be used to enhance the efficacy of *Trichoderma* in the control of *Pythium*.

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Biocontrol agents and composts suppress Fusarium and Pythium root rots on greenhouse cucumbers

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Abstract: Two important root pathogens of greenhouse cucumbers in British Columbia, Canada, are Fusarium root and stem rot (RSR), (*F. oxysporum* f.sp. *radicis-cucumerinum*) and Pythium damping-off and crown rot (DO) (*Pythium aphanidermatum*). Three composts (greenhouse waste, windrow dairy solids, vermicompost dairy solids) were evaluated for their ability to reduce RSR, and several commercially available biocontrol agents (BCAs) were evaluated for their ability to reduce RSR and DO. Seedlings were grown in rockwool blocks that received either compost or BCA in the germination plug at time of seeding. All 3 composts reduced RSR to some degree, with greenhouse waste compost reducing mortality significantly by 50%. Autoclaved compost had no effect on disease suppression, suggesting that microbial antagonism was involved. The most effective BCA that reduced RSR and DO was *Gliocladium catenulatum* strain J1446 (PreStop, PreStop Mix), while *Streptomyces griseoviridis* strain K61 (MycoStop) also reduced the severity of these diseases to some degree. *Trichoderma harzianum* strain T-22 (Plant Shield) and *T. virens* strain GL-21 (Soil Gard) had no significant effect. Under semicommercial greenhouse propagation trials, PreStop WP, Plant Shield, MycoStop and windrow composted dairy solids reduced FORC in two out of three trials. PreStop and MycoStop also reduced the severity of DO due to *Pythium*. The efficacy of the BCA was affected by seasonal differences in growing conditions (temperature, relative humidity, light intensity), which affected the incidence and severity of the diseases.

Introduction

Greenhouse cucumbers (Long English type) are grown under approximately 200 ha of glasshouse in British Columbia using hydroponic systems. The unique growing substrate (rockwool) used in most greenhouses presents both challenges and opportunities for management of plant diseases. Establishment of competing micro-organisms, including biocontrol agents, can be challenging. The greenhouse environment, however, is environmentally controlled, contains an economically high-value crop, and can present opportunities for implementation of biocontrol to disease management (Paulitz & Belanger, 2001). Two root-infection pathogens of importance in commercial greenhouse cucumber production in British Columbia are *Fusarium oxysporum* f.sp. *radicis-cucumerinum* (FORC), cause of root and stem rot (RSR) (Punja & Parker, 2000), and *Pythium aphanidermatum*, cause of damping-off and crown rot (DO). At present, no fungicides are registered for use against these two diseases, and in the absence of genetically resistant cultivars, growers are left with few options for disease management other than cultural control practices, including sanitation. The objectives of this research were to evaluate the effects of amendments to the rockwool growing medium, consisting of composts or commercially available BCAs which are registered in the USA/Europe, on severity of RSR and DO.

Materials and methods

Macro- and micro-conidia of *Fusarium* (10^5 spores/ml) from 2-week-old PDA cultures and blended mycelial mats of *Pythium* (10^4 cfu/ml) from 10-day-old broth cultures were used as inoculum. Cucumber seeds were placed in the germination cavity of rockwool blocks, followed by a compost or biocontrol treatment, and inoculated with *Fusarium* after 48 hrs or *Pythium* after 10 days. Plants inoculated with *Fusarium* were maintained at 24±2°C and those receiving *Pythium* at 30±2°C for 30 days in growth chambers (14-hrs photoperiod). There were a minimum of 10 plants/treatment. We measured plant mortality, plant height and fresh weight.

Three composts were evaluated against *Fusarium* - (i) from greenhouse plant waste material (GC); (ii) from windrow composted dairy solids (WDS); (iii) from vermicomposted dairy solids (VMC) (Kannangara et al., 2000). The BCAs were drenched as follows: *Trichoderma harzianum* (Plant Shield), 0.9 g/L, 5 ml/plant; *Streptomyces griseoviridis,* 1.0 g/L, 20 ml/plant; *Gliocladium catenulatum* (PreStop WP, PreStop Mix), 10 g/L, 15 ml/plant. Granules of *Trichoderma virens* (SoilGard) were mixed in vermiculite (0.6 g/L) and 2.5 cm^3 was added to the seeding cavity. Control plants received vermiculite only (pathogen-free), pathogen only, and biocontrol treatment only (pathogen-free), or fungicide with pathogen (benomyl 50% a.i., 12.5 ml/plant).

Three trials were conducted in a semi-commercial greenhouse to evaluate the BCAs WP and WDS for disease control due to *Fusarium.* Two trials were conducted for *Pythium.* Plant mortality and plant height assessments were used to calculate a Disease Severity Index (Punja & Parker, 2000). Trials were conducted using a completely randomized design with 10 replications and trials were conducted twice. Data were analyzed using ANOVA and treatment means separated with Tukey-Kramer HSD at $P \leq 0.05$.

Results

Greenhouse compost significantly reduced plant mortality due to *Fusarium* (Figure 1), averaging 13% compared to 63% for FORC alone over three trials. Both WDC and VMC composts reduced plant mortality but these values were not statistically different from the pathogen control (Figure 1). Autoclaved GC had no disease-suppressive effect. Benomyl provided a level of disease control similar to GC. The biocontrol agent *G. catenulatum* (PreStop, PreStop Mix) significantly reduced mortality due to *Fusarium* (Figure 2), while neither MycoStop, Plantshield nor SoilGard provided protection from infection under the conditions of this experiment. For *Pythium,* PreStop significantly enhanced plant survival and was the only treatment that was effective under these conditions. The semi-commercial trials conducted for *Fusarium* control showed that disease severity varied according to the season of growth (Table 1). In trial 1, there were no significant differences among any of the treatments; in trial 2, PreStop WP, WDS compost, Plantshield and MycoStop all significantly reduced DSI; in trial 3, all treatments also significantly reduced DSI. In the trials for *Pythium* control, PreStop WP, PreStop Mix and MycoStop all significantly reduced disease severity (Figure 3).

Discussion

Composts have received considerable interest for their disease-suppressive activities (Hoitink & Boehm, 1999). Both general and specific mechanisms of disease suppression are involved, and include microbial antagonism, nutrient release, induced host resistance, and a-biotic

inhibitory factors. Our results demonstrated that composts added at seeding time to rockwool medium reduced *Fusarium* infection, potentially through microbial antagonism.

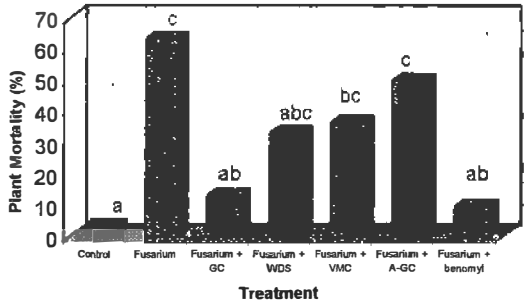

Figure 1. Effect of greenhouse compost (GC), windrow composted dairy solids (WDS), vermicomposted dairy solids (VMC), autoclaved greenhouse compost (A-GC) and the fungicide benomyl on mortality of cucumber seedlings due to *F. oxysporum* f.sp. *radicis-cucumerinum*. Columns followed by different letters are significantly different ($P \leq 0.05$, Tukey-Kramer's HSD test).

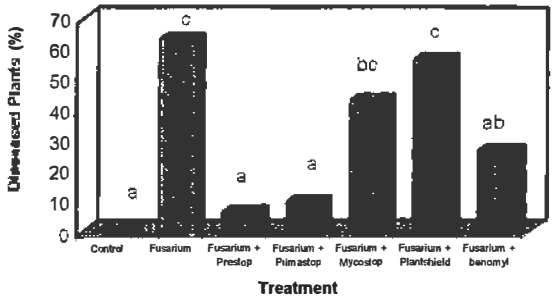

Figure 2. Effect of the biocontrol agents Prestop WP and Prestop Mix (*Gliocladium catenulatum)*, Mycostop (*Streptomyces griseoviridis*), Plantshield (*Trichoderma harzianum*) and the fungicide benomyl on mortality of cucumber seedlings due to *F. oxysporum* f.sp. *radicis-cucumerinum*. Columns followed by different letters are significantly different ($P \leq 0.05$, Tukey-Kramer's HSD test).

Table 1. Effect of three biocontrol agents and windrow composted dairy solids on development of *FORC* on cucumber seedlings grown under semi-commercial propagation conditions

	Disease Severity Index (DSI) [x]		
	Trial 1 [y]	Trial 2 [z]	Trial 3 [z]
Treatment	July-August 01	August-September 01	February-March 02
Uninoculated control	0.00	0.00 [a]	0.00 [ab]
Fusarium *only*	0.26	3.27 [c]	1.31 [d]
Prestop WP + *Fusarium*	0.16	0.43 [ab]	0.21 [c]
Plantshield + *Fusarium*	0.07	0.59 [ab]	0.16 [c]
Mycostop + *Fusarium*	0.09	0.81 [b]	0.05 [ab]
Compost + *Fusarium*	0.03	0.29 [ab]	0.04 [a]
Benomyl + *Fusarium*	0.01	0.00 [a]	0.06 [b]

[x] Disease severity index (DSI) were calculated as described in the Materials and methods.
[y] Trial 1 represents the combined data from two separate trials (June-July, July-August 2001) in which there were no significant differences among any of the treatments in either trial.
[z] Means are the average of 10 replications, and when followed by the same letter, are not significantly different ($P \leq 0.05$, Tukey-Kramer's HSD test).

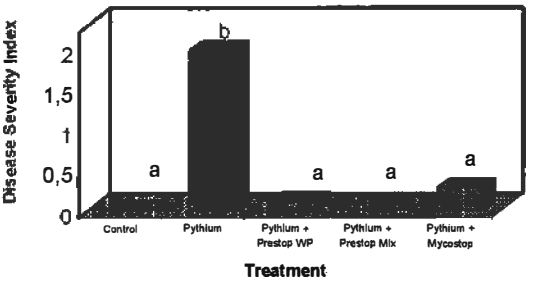

Figure 3. Effect of two biocontrol agents on development of *Pythium aphanidermatum* on cucumber seedlings grown under semi-commercial propagation conditions.

Not all the composts tested were effective. Variability within and between composts is a factor for consideration before widespread implementation of this approach to disease management is achieved. BCAs tested in growth chamber and semi-commercial greenhouse trials demonstrated differing efficacy due to differences in disease pressure brought on by differing environmental conditions. Under high disease pressure in the growth chamber, only *G. catenulatum* significantly reduced RSR and DO. Under greenhouse conditions, low to moderate disease pressure revealed that *G. catenulatum, S. griseoviridis* and *T. harzianum* were all effective. MycoStop is currently the only BCA registered for disease control, mainly due to *Pythium*, in greenhouses in Canada. Our results demonstrated that *G. catenulatum* has greater potential for control of root rot pathogens on cucumber, and extends the results reported by McQuilken et al. (2001) on this BCA.

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Influence of synthetic pesticides on the effectiveness of the *Coniothyrium minitans* based bio-fungicide Contans®WG

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Abstract: The market introduction of the *Coniothyrium minitans* based bio-fungicide Contans®WG for the control of *Sclerotinia* diseases in oilseed rape, sunflower, soy bean, field vegetables and other important crops requires a detailed knowledge about the compatibility of the beneficial fungus with other plant protection compounds in the soil. The active compound of Contans®WG, the soil fungus *C. minitans*, is formulated and applied as living micro-organism as a pre-plant or pre-sowing application. The assumption was that fungal toxic effects to the conidia of *C. minitans* can occur directly while applied as tank mixture with pre-sowing herbicides (Trifluraline, Napropamide) or indirectly when pre-emergence or post-emergence pesticides or seed staining were used after Contans®WG was applied into the soil and will reduce the sclerotia degrading effectiveness of the preparation. Additionally the potential combination of the bio-fungicide and synthetic pesticides for the application as tank mixtures would simplify the usage of Contans®WG and increase its acceptance on and integration into the conventional market. For the detection of the compatibility of Contans®WG with synthetic pesticides a two stepped test system was developed consisting on *in vitro* laboratory tests and an *in situ* greenhouse biotest. While the *in vitro* tests showed the direct fungicide effects of the tested preparations to *C. minitans* by using modified agar diffusion tests the greenhouse biotest worked with non-sterile field soil and simulated the real conditions of the usage of Contans®WG and its potential combination partners. The presentation will show results from both test systems that actually includes data of more than 100 different preparations. The discussion will focus on the apparently contradictory results of the *in vitro* and *in situ* tests and emphasize the necessity investigating the influence of synthetic crop protection agents to biocontrol agents.

Biocontrol and resident micro-organisms

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Use of GUS transformants of *Trichoderma harzianum* isolate T39 (TRICHODEX) for studying interactions on leaf surfaces

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Abstract: *Trichoderma harzianum* isolate T39 (TRICHODEX), was transformed with the GUS (ß-glucuronidase) reporter gene to visualize the interaction with *Botrytis cinerea* and to determine potential mechanisms involved in biocontrol. T39GUS transformants did not appear to penetrate the bean leaf epidermis or to grow into the mesophyll via stomata. No direct penetration or coiling was observed between hyphae and conidia of the T39GUS transformants and of *B. cinerea*, however structures of the pathogen were stained blue. Similarly, blue substrate accumulated in various non-transformed fungi such as *Alternaria* sp., *Colletotricum acutatum* and the wild-type isolate T39 when co-inoculated on leaves and plates with the T39GUS-transgenic strains of *T. harzianum* and *Fusarium oxysporum* f. sp. *niveum*. Therefore, the role of diffusible compounds, specifically from *T. harzianum* transformants, will need further investigation.

Keywords: Biocontrol mechanisms, *Botrytis cinerea*, Grey mould, GUS-transformants, *Trichoderma harzianum*, TRICHODEX

Introduction

Trichoderma spp. are commonly used in biocontrol of plant pathogens (Elad & Freeman, 2001). *T. harzianum* T39, the active ingredient of the commercial preparation TRICHODEX, has been used for the control of foliar diseases such as grey mould in various crops, and other pathogens of cucumber and tomato (Elad, 2000). Prevention of infection by biocontrol agents or suppression of disease is based on induced resistance in the host plant, competition with the pathogen for nutrients and space, antibiosis, hyperparasitism, reduction of the saprophytic ability and inhibition of spore dissemination and/or restraining of pathogenicity factors of the pathogen (Elad & Freeman, 2002). T39-related biocontrol was not regarded as being achieved by means of antibiotics or by mycoparasitism (Elad, 2000). T39 was found to restrain enzymes of *Botrytis cinerea*: the activities of exo- and endo-polygalacturonase, pectin methyl esterase, pectate lyase, chitinase, β-1,3-glucanase and cutinase of *B. cinerea*, were reduced on bean leaves by T39 (Kapat et al., 1998). T39 has been found to manifest a direct effect on enzymes of *B. cinerea* by secretion of protease in the presence of the pathogen, thus, spore germination and subsequent disease development are suppressed (Elad & Kapat, 1999).

Transgenic *Trichoderma* isolates, harbouring the GUS transporter gene, have been used for numerous studies. Population survival studies, measurement of conidial germination and mycelium growth rates were monitored using GUS-transformed isolate 3a of *T. harzianum* in the rhizosphere of cucumber-grown plants (Green & Jensen, 1995). Likewise, ecological studies were conducted using GUS-marked strains of *T. harzianum* in the rhizosphere (Green et al., 2001). In this study, we used GUS-transformants of *T. harzianum*, isolate T39 to (i) determine the potential uptake of compounds into nontransformed fungi released from the marked strains and (ii) attempt to confirm mode of interaction with *B. cinerea* and other fungi in bean.

Materials and methods

Isolates and inoculation assays

Isolate T39 was maintained on PDA at 25°C and transformed by the polyethylene glycol-mediated protoplast protocol with a plasmid (pGPD-GUS; kindly provided by D. Straney, Univ. of Maryland, USA) containing the GUS (ß-glucuronidase)-reporter gene and resistance to hygromycin (100 µg/ml) (Freeman et al., 1999). Interaction of the T39GUS-transgenic isolates and their biocontrol ability were studied in co-inoculation studies with pathogenic isolate BcI16 of *B. cinerea* (Kapat et al., 1998), isolate TUT-5954 of *Colletotrichum acutatum* pathogenic on strawberry (Freeman & Katan, 1997) and a GUS-transformed isolate FUS-GUS-8 of *Fusarium oxysporum* f. sp. *niveum* pathogenic on watermelon. Drops containing conidial suspensions of BcI16 and T39 were adjusted to 10^5 and 10^6 conidia/ml for disease and germination tests, respectively (Kapat et al., 1998). Disease severity was evaluated on the bean leaves during 8-9 days, determined visually using a 0-100% scale of rot coverage (Guetsky et al., 2001).

Histopathological studies and GUS activity

Histopathological studies and GUS activity of the interactions between the various fungal strains were observed microscopically either by co-inoculating bean leaves or placing inoculum in 5-mm-diameter well slides. Conidia of transformants and the non-transformed wild-type isolates were suspended in 0.2% glucose and casein hydrolysate solutions. Co-inoculation experiments were conducted by placing 10 µl drops of conidial suspensions (10^6 conidia/ml of T39, *Colletotrichum* and *Fusarium* and $3X10^5$ conidia/ml of isolate BcI16) on bean leaves or slides. GUS-stained mycelium were detected in a 10 µl drop of reagent mix which was added to the fungus and incubated for 2-hr at 37°C before observation (Freeman et al., 1999). Bean leaf tissues were cleared of chlorophyll by washing twice over a 48-hrs period in chloral hydrate (120 g/100 ml) and viewed using a Wild stereomicroscope (Freeman et al., 1999).

Results and discussion

Ten stable hygromycin-resistant transformants of T-39 were obtained. Three of the transformants (T39GUS-3, -5, -12) and wild-type T39 were used to assess the effect on germination and biocontrol of *B. cinerea* on detached bean leaves. Germination of *B. cinerea* conidia was 100% after 7 hours in the control leaves, compared to 0 to 20% in the various T39GUS transformants. The T39GUS transformants suppressed *B. cinerea* and did not differ from the original T39 isolate in respect to effect on germination of *B. cinerea* conidia and disease reduction, (Figure 1). No significant differences were recorded in germination percent between the wild-type T39 isolate and that of the transformants (data not shown). All isolates reduced disease to a similar extent, without significant differences between them ($P \leq 0.05$).

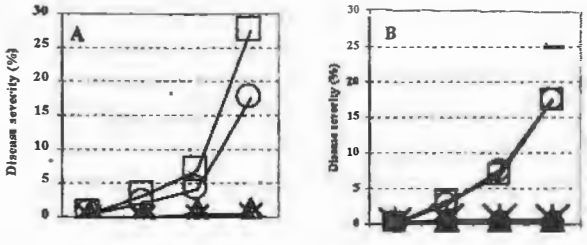

Figure 1. Effect of wild type (A) and transformant T39GUS-3 (B) of *Trichoderma harzianum* T39 (*T.h.*) on disease severity of *Botrytis cinerea (B.cin) on* detached bean leaves. Treatments were *B.cin* alone (O,□); *T.h.*+*B.cin.* (Δ,*); and *T.h.* alone (◊). No significant differences were observed when comparing disease reduction between the T39GUS transformants and wild type isolate of T39 ($P \leq 0.05$).

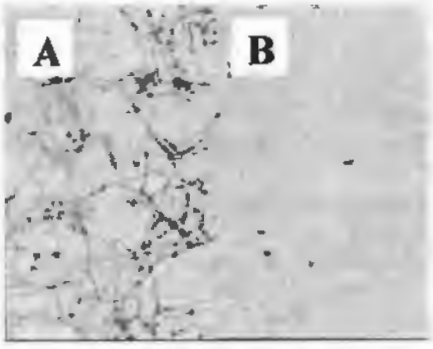

Figure 2. Localization of GUS activity in transformant T39GUS after co-inoculation with isolate BcI16 (thick hyphae stained blue) - A; no GUS staining in a wildtype T39 and isolate BcI16 - B.

Typical dark blue staining was observed in hyphae and conidia of all transformed isolates and not in that of the control wild-type isolate T39. In co-inoculation studies with transgenic T39GUS isolates and isolate BcI16 of *B. cinerea* on slides and on bean leaves (Figure 2A), no penetration or coiling between the GUS-stained hyphae and that of the pathogen was observed. However, conidia and hyphae of *B. cinerea* were stained blue, although to a lesser extent than that of the transgenic GUS isolates. Isolate BcI16 of *B. cinerea* did not stain blue in the presence of non-transformed isolate T39, neither on slides nor on leaves (Figure 2B). In a similar experiment on glass slides and leaves, isolate T39GUS-12 caused staining of conidia and mycelium of *C. acutatum*, *Alternaria* sp. and wild-type isoate T-39, without any visible interaction between the fungi (data not shown).

Interference with pathogenicity processes has only recently been studied in *T. harzianum* T39 on germination and subsequent lesion production by *B. cinerea* on bean leaves. The activities of PG, PME, PL, chitinase, β-1,3-glucanase and cutinase produced by *B. cinerea* was reduced in the presence of T39 (Kapat et al., 1998). Cysteine protease was found responsible for deactivation of pathogenicity enzymes (Elad et al., 1998). In this study, we observed that the T39GUS transformants were able to actively stain other non-transformed fungal hyphae and conidia that grew in close proximity, although penetration and coiling of the two types of hyphae did not occur (Figure 2A). However, enzymes produced by the

biocontrol agent have the potential to diffuse in the film of water present on the leaf surface and to reach the pathogen. This indicates that certain metabolites originating from the control agent may reach hyphae of the pathogen. Similar phenomena may take place with compounds that are important for biocontrol. T39GUS transformants of T39 also stained wild-type isolate T39 but did not cause any visible damage to the fungus, suggesting that T39 germinating conidia can affect each other via diffusible materials on the leaf surface. The potential of *T. harzianum* isolate T39 to affect other fungi on leaf surfaces by releasing active compounds needs further elucidation.

Acknowledgement

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How soilborne fungal pathogens affect the production of 2,4-diacetylphloroglucinol in biocontrol strain *Pseudomonas fluorescens* CHA0

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Abstract: 2,4-Diacetylphloroglucinol (Phl) is one of the most important factors in the biocontrol of soilborne pathogens by fluorescent pseudomonads. Many Phl producing strains have been shown to be effective against several plant diseases in laboratory and greenhouse experiments. However, the performance of these strains in the field is quite inconsistent probably due to biotic and a-biotic environmental factors influencing Phl production and biocontrol in these bacteria. We have investigated the influence of various soilborne fungi on the expression of a Phl biosynthetic gene in biocontrol strain *Pseudomonas fluorescens* CHA0 in vitro and in the rhizosphere of different plant species grown in a gnotobiotic system. For these experiments a *lacZ* reporter gene translationally fused to the promoter of the Phl biosynthetic gene *phlA* was used. We found that culture filtrates of the pathogen *Pythium ultimum* enhanced *phlA'-'lacZ* expression in CHA0 grown in liquid cultures. The presence of *P. ultimum* also enhanced the expression of the *phlA* gene several-fold in the rhizosphere of cucumber, wheat and maize. Culture filtrates of different *Fusarium oxysporum* strains producing the fungal pathogenicity factor fusaric acid inhibited *phlA'-'lacZ* expression *in vitro* and one fusaric acid producing strain also had a negative effect on expression of the Phl biosynthetic gene in the rhizosphere of wheat. Interestingly a non-pathogenic *F. oxysporum* strain, which does not produce fusaric acid, had no influence on *phlA* expression in liquid cultures and even enhanced the expression of this gene in the wheat rhizosphere. Other root pathogens like *Thielaviopsis basicola* and *Gaeumannomyces graminis* var. *tritici* did not influence *phlA* expression in CHA0 in the rhizosphere of tobacco and wheat, respectively. We conclude that indeed soilborne fungal pathogens can have an impact, negative or positive, on the production of those metabolites that are key factors in biocontrol by fluorescent pseudomonads.

Introduction

Biocontrol of soilborne plant pathogens by the use of beneficial bacteria is a promising alternative to the use of pesticides. The performance of biocontrol strains in the field, however, tends to be inconsistent and varies between different years and application sites. There is evidence that at least part of this variability is caused by the influence of environmental factors on the production of those bacterial metabolites, which play an important role in disease suppression. The polyketide compound 2,4-diacetylphloroglucinol (Phl) has emerged as one of the most important antimicrobial compounds produced by biocontrol strains of *Pseudomonas fluorescens*. Several studies have shown that certain a-biotic factors (e.g., mineral concentrations) and biotic factors such as plant species, plant age and fungal pathogens effect the production of Phl in the well characterised biocontrol agent *P. fluorescens* CHA0 (Duffy & Défago, 1997; Notz et al., 2001; 2002). Here we present an

overview on the effects of different soilborne plant pathogens on the expression of a Phl biosynthetic gene in strain CHA0.

Material and methods

Microorganisms and culture conditions

A derivative of *P. fluorescens* strain CHA0 harbouring plasmid CHA0/pME6259 containing a translational *phlA'-'lacZ* fusion was used to measure the expression of the Phl biosynthetic gene *phlA*. Bacteria were routinely cultivated on King's B agar or Luria-Bertani broth (LB) at 27°C. *Pythium ultimum* 67-1, *Thielaviopsis basicola* D127, *Gaeumannomyces graminis* var. *tritici* ETH1000, *Fusarium oxysporum* f. sp. *radicis lycopersici* 22 and *F. oxysporum* strains 249, 241, 242, 410, 798 and 801 were routinely cultivated on malt agar at 24°C.

Influence of fungal culture filtrates on phlA'-' lacZ expression in liquid cultures

P. ultimum, *T. basicola* and *G. graminis* var. *tritici* were grown in liquid malt medium at 24°C for 14 days. Two ml of filtrates (filter pore size = 0.8 µm) obtained from these cultures were added to 18 ml glycerol-casamioacids-medium (GCM) and the media was inoculated with 20 µl of an overnight LB culture of strain CHA0/pME6259. Bacterial cultures were grown for 24 hrs at 27°C and samples were taken at different time intervals to determine bacterial growth and β-galactosidase activity as a measurement of *phlA* expression. Experiments with culture filtrates of *F. oxysporum* strains have been described elsewhere (Notz et al., 2002).

Influence of fungal pathogens on phlA expression in the rhizosphere

All these experiments have been performed in gnotobiotic systems with artificial soil. These systems have already been described elsewhere in (Notz et al., 2001; Maurhofer et al., 1995; Duffy & Défago, 1997; Notz et al., 2002). CHA0/pME6259 was added to the gnotobiotic system by soaking seedlings in a bacterial suspension containing 10^7 cfu/ml for 1 hr prior to transplanting them into the artificial soil, which was inoculated with different fungal pathogens. At the end of the experiments, plants were harvested and the roots were assessed for bacterial colonisation and β-galactosidase activity as described by Notz et al. (2001).

Results and discussion

Culture filtrates of *P. ultimum* and *G. graminis* var. *tritici* both enhanced expression of *phlA* in GCM liquid cultures whereas culture filtrates of *T. basicola* did not have any effect (Figure 1). The stimulating effect of the culture filtrates was transient and disappeared when bacterial cultures reached on optical density (OD, at 600 nm) of about 2.5. What compounds in the fungal filtrates may have caused the induction of gene expression is not yet known.

Culture filtrates of *F. oxysporum* strains which contained FA inhibited *phlA'-'lacZ* expression in Czapek Dox medium (Figure 2). It is known that synthetic FA inhibits the production of Phl in CHA0 (Duffy & Défago, 1997). Since the degree of *phlA*-repression correlated with the amount of FA present in the different treatments, we conclude that FA produced by the *Fusarium* strains was responsible for this effect.

In the rhizosphere the presence of *P. ultimum* had a significantly enhancing effect on *phlA* expression in all three plant-pathogen system tested (Table 1). This could either be due to some signal compounds released by this fungus or, what we think is rather the case, due to some compounds released by the wounded plants, which have a stimulating effect on the expression of the Phl biosynthetic gene. *G. graminis* var. *tritici* and *T. basicola* had no significant effects on *phlA* expression (Table 1).

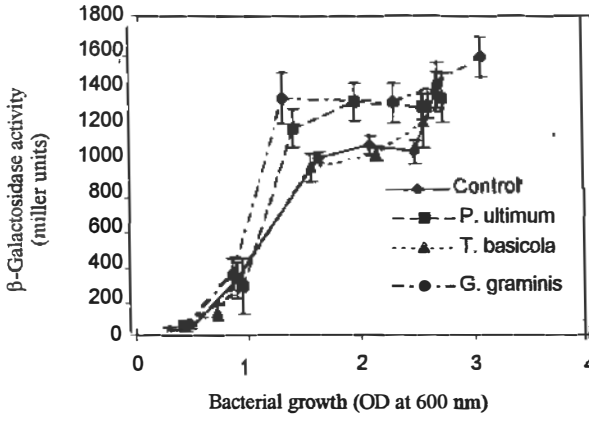

Figure 1. Effect of culture filtrates of three different fungal pathogens (grown in liquid malt medium for 14 days) on the expression of a *phlA'-'lacZ* translational fusion in *P. fluorescens* CHA0 grown in GCM medium at 27°C. Filtrates of the three strains were added to the growth medium at a ratio of 1:9. Control treatments received the same amount of malt medium. Throughout growth, β-galactosidase activities and OD_{600} were determined. Values are the means of three replicate cultures.

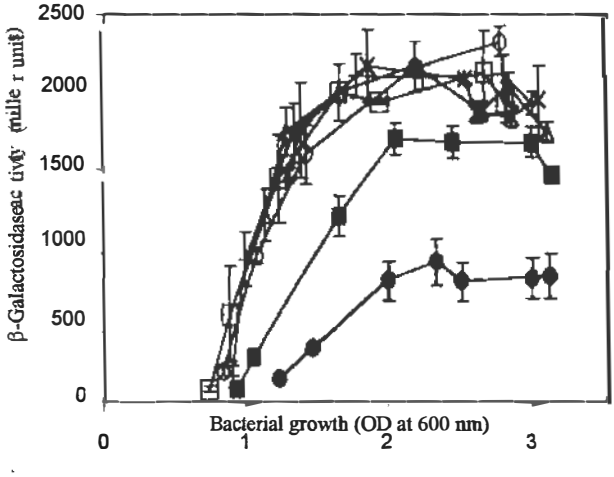

Figure 2. Effect of culture filtrates of six different *F. oxysporum* strains (grown in Czapek Dox for 7 days) on the expression of a *phlA'-'lacZ* translational fusion in *P. fluorescens* CHA0 grown in OSG medium at 24°C. Filtrates of the six *F. oxysporum* strains were added to the medium at a ratio of 1:4 which gave the following final fusaric acid concentrations of: 240 (□) (0 μM), 241 (x) (23 μM), 242 (▫) (0 μM), 410 (+) (0 μM), 798 (●) (698 μM), and 801 (■) (157 μM). (○) = control. Values are the means of three replicate cultures.

When adding a FA producing *Fusarium* strain either to tomato or to wheat seedlings inoculated with the *phlA'-'lacZ* reporter strain, the expression of *phlA* in the rhizosphere was significantly repressed compared to control treatments treated with strain CHA0/pME6259 alone (Table 1). These results confirm the findings of Duffy & Défago (1997), who demonstrated an inhibiting effect of FA on Phl production. Interestingly the FA-negative *F. oxysporum* strain 242, which is non-pathogenic to wheat, increased *phlA* expression in the wheat rhizosphere several-fold (Table 1). We suggest that since in this system no pathogenic interactions have been observed, the change in gene expression is probably due to a signal produced by the fungus rather than by the plant. Taken together our results show, that indeed the presence of fungal pathogens can, positively or negatively, affect the expression of a gene, which is of importance in the suppression of soilborne diseases by fluorescent *Pseudomonas*.

Table 1. Differential influence of soilborne plant pathogens on the expression of a 2,4-diacetylphloroglucinol biosynthetic gene in *P. fluorescens* CHA0 in the rhizosphere

Plant-pathogen system[y]	*PhlA* expression in CHA0 (β-galactosidase activity in units/10^8 cfu)	
	without pathogen	with pathogen
Cucumber-*P. ultimum*	3.0 a[z]	10 b
Maize-*P. ultimum*	45 a	106 b
Wheat-*P. ultimum*	140 a	647 b
Wheat-*G. graminis* var. *tritici*	205 a	140 a
Tobacco-*T. basicola*	25 a	31 a
Tomato-*F. oxysporum* f. sp. *radicis-lycopersici* (FA^+)	132 a	65 b
Wheat- *F. oxysporum* strain 242 (FA^-)	65 a	304 b
Wheat- *F. oxysporum* strain 798 (FA^+)	65 a	38 b

[y] Seedlings were inoculated with strain CHA0/pME6259 (10^7 cfu/ml) carrying a translational *phlA'-'lacZ* fusion and grown in a gnotobiotic system for 7 (wheat-*Fusarium*), 14 (cucumber, maize, tomato) or 21 days (tobacco, wheat-*G. graminis* var. *tritici*). Pathogens were inoculated into the soil before adding plants. At the end of the experiments roots were assessed for β-galactosidase activity and bacterial colonisation.

[z] Experiments have been repeated three times. One representative experiment is presented. Values in the same row followed by the same letter are not significantly different at $P = 0.05$ (Fishers' protected LSD). Part of these results has already been published (Notz et al., 2001; 2002).

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Dose response relationships for control of fusarium wilts by *Pseudomonas fluorescens* RS111

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Abstract: A spontaneous mutant of *Pseudomonas fluorescens* RS111 that is less sensitive to antagonism by other strains of fluorescent pseudomonads was isolated. This mutant, designated RS111a, appeared to be more effective in suppression of fusarium wilt than the parental strain. In this study we investigate dose response curves for both RS111 and RS111a in the suppression of fusarium wilt in radish and flax. Mutant banks of both strains were generated by transposon mutagenesis to identify bacterial traits involved in disease suppression.

Key words: antifungal compounds, *Fusarium oxysporum*, induced systemic resistance, ISR, *Pseudomonas fluorescens*, siderophores

Introduction

Fusarium wilt diseases, caused by the fungus *Fusarium oxysporum*, lead to significant yield losses of horticultural and agricultural crops. Possibilities to manage fusarium wilt are limited. Therefore, other strategies to control the disease, such as biocontrol, are being developed. The use of combinations of strains of antagonistic fluorescent *Pseudomonas* spp. to achieve reliable biocontrol of fusarium wilt was described by De Boer et al. (1999). Their study suggested that specific interactions between the biocontrol strains influence the level of disease suppression by combinations of these strains. *P. fluorescens* strain RS111 shows high levels of disease suppression, however, in combination with other strains disease control is less efficient. Strain RS111 is very sensitive to antagonism by other fluorescent pseudomonads. A spontaneous mutant of RS111, designated RS111a, that is much less sensitive to antagonism by other *Pseudomonas* strains, was isolated. In some combinations with other biocontrol pseudomonads RS111a suppressed disease significantly better than RS111 (De Boer et al., 1999). In dose-response experiments (Raaijmakers et al., 1995) RS111a suppressed disease already at much lower population densities than the wild type RS111 (De Boer, 2000).

In the present study we investigated dose response curves of RS111 and RS111a not only in the radish – *F. oxysporum* f.sp. *raphani* system, but also on flax with *F. oxysporum* f.sp. *lini* as the pathogen. In the radish system involvement of induced systemic resistance (ISR) in disease suppression was studied. To identify bacterial determinants that play a role in suppression of fusarium wilt a Tn*5* transposon insertion mutant bank was generated and screened for mutants defective in production of the pseudobactin siderophore or antifungal compounds.

Materials and methods

Microbial strains

P. fluorescens strain RS111 and its derivative RS111a (De Boer et al., 1999) were used. The fungal strains used were *F. oxysporum* f.sp. *raphani* WCS600, pathogenic on radish (Leeman et al., 1995) and *F. oxysporum* f.sp. *lini* Foln3, pathogenic on flax (Lemanceau & Alabouvette, 1991).

Dose-response experiments

The assays with radish were performed as described by De Boer et al. (1999) and those with flax as described by De Boer (2000). Conidia of the pathogens were mixed through soil (a mixture of potting soil and river sand) and this inoculated soil was mixed with soil containing different population densities of RS111 or RS111a. The final population densities of the bacteria were 10^2, 10^4, 10^6, and 10^8 colony forming units (cfu) per gram of soil.

Induced systemic resistance assays

The bioassay described by Leeman et al. (1995) was used to study the involvement of ISR in suppression of fusarium wilt. In this bioassay the pathogen and the biocontrol agent are kept spatially separated on the root system of the plant to avoid direct interactions between the two populations. Strains WCS358 and WCS417 were used as a negative and a positive control, respectively (Leeman et al., 1995).

Transposon mutagenesis and mutant screening

To perform random mutagenesis of *P. fluorescens* RS111 and RS111a, the mobilization system of *Escherichia coli* S17-1 and the suicide vector pSUP2011, carrying transposon Tn*5*, were used (Simon et al., 1983). Siderophore production by bacterial strains was detected by using the universal siderophore detection medium CAS agar (Schwyn & Neilands, 1987), as described by Mercado-Blanco et al. (2001). Production of antifungal compounds by the bacteria was detected on agar medium with high iron availability. Bacterial colonies were grown and subsequently the agar plates were sprayed with spore suspensions of the fusarium strains.

Results and discussion

In both radish and flax effects of different population densities of RS111 and RS111a on disease development were studied. In radish all bacterial treatments suppressed disease; however, RS111a was more effective than RS111, especially at the lower population densities. These results confirm those of De Boer (2000). In flax RS111 was only effective at the higher population densities (10^6 and 10^8 cfu/g soil), whereas RS111a was also effective at 10^2 and 10^4 cfu/g soil and even more effective at the higher densities.

In the ISR bioassay WCS358 did not reduce disease, whereas WCS417 did. This confirms the results obtained by Leeman et al. (1995). Both RS111 and RS111a suppressed disease significantly in this bioassay, however, RS111a was more effective than RS111 (Table 1). In *Arabidopsis thaliana* two signal tranduction pathways for induced systemic resistance have been described (Van Loon et al., 1998). One depends on accumulation of salicylic acid and is induced by pathogen infection (SAR), whereas ISR induced by rhizobacteria is independent of SA accumulation, but depends on intact responses to jasmonic acid and ethylene (Pieterse et al., 1998). Van Wees et al. (2000) demonstrated that simultaneous activation of the SAR and the ISR pathway can lead to enhanced induced disease resistance. We speculate that RS111 induces only one signal transduction pathway leading to systemic resistance, whereas RS111a induces more than one pathway.

Table 1. Induced systemic resistance against fusarium wilt in radish by different strains of fluorescent *Pseudomonas* spp.

Treatment	Diseased plants (%)
Control	48
WCS358	45
WCS417	26
RS111	27
RS111a	15

To further unravel the modes of action of the two strains Tn*5* transposon mutant banks of RS111 and RS111a were generated. Both strains produce an antifungal compound and non-producing mutants were identified. It was noted that the antifungal activity of RS111a was much less compared to that of RS111. This observation is opposite to what was found for suppression of disease by the two strains and we speculate that the antifungal factor is not very important in disease suppression. We also detected a difference between RS111 and RS111a in siderophore production. On CAS medium non-fluorescent, pseudobactin mutants of RS111 still produce halos, indicating that RS111 produces more than one siderophore. On the other hand RS111a only produces the pseudobactin siderophore, since non fluorescent mutants no longer produce halos on CAS medium. The presence of a non fluorescent siderophore in *P. fluorescens* has been reported earlier (Mercado-Blanco et al., 2001). The different mutants of RS111 and RS111a are currently being tested for their abilities to suppress disease.

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Biocontrol agent *Serratia plymuthica* strain HRO-C48: Performance in relation to environmental factors

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Abstract: *Serratia plymuthica* strain HRO-C48 was evaluated for plant growth promotion of strawberries and biocontrol of the fungal pathogens *Verticillium dahliae* and *Phytophthora cactorum*. The antifungal activity of the strain mainly based on an efficient chitinolytic system. In three consecutive vegetation periods, field trials were carried out in soils naturally infested by the soilborne pathogens. Dipping plants in a suspension of *S. plymuthica* prior planting reduced Verticillium wilt compared with the nontreated control by 0 to 37.7%, with an average of 24.2%, whereas the increase of yield ranged from 156 to 394%, with an average of 296%. *Serratia* treatment reduced Phytophthora root rot by 1.3 to 17.9%, with an average of 9.6%, and increased strawberry yield by 60% compared with the nontreated control. The results of the field trials were influenced by a-biotic conditions, e.g., weather and soil quality and by biotic conditions, e.g., pathogen inoculum density and autochthonous microflora. Additionally, influence of the biocontrol agent on the bacterial communities (non-target microorganisms) of the strawberry rhizosphere was monitored by analysis of PCR-amplified fragments of the 16S rRNA genes of the whole bacterial community after separation by denaturing gradient gel electrophoresis (DGGE). The DGGE pattern of rhizosphere communities did not show differences between the treated and the nontreated strawberries. Under field conditions, the strain survived at approximately $\log_{10}$ 3-7 CFU/g root in the strawberry rhizosphere at 14 months after root application.

Keywords: *Serratia plymuthica, Verticillium, Phytophthora*, strawberry, biocontrol

Introduction

In recent years, an increase in strawberry (*Fragaria x ananassa*) production has been recorded worldwide (FAO, Statistical Databases). Verticillium wilt caused by *Verticillium dahliae*, and root rot caused by *Phytophthora cactorum* are important diseases in commercial production. In the coming years, the loss of methyl bromide as a control measure for Verticillium wilt will have a great impact on the accumulation of microsclerotia in soil. An environmentally friendly alternative to protect roots against fungal pathogens is rhizobacteria-mediated biocontrol. *Serratia plymuthica* strain HRO-C48 was isolated from the rhizosphere of oilseed rape and selected as a biocontrol agent according to the following criteria: (a) high chitinolytic activity responsible for activity against fungal pathogens such as *V. dahliae* and *P. cactorum in vitro* (Berg et al., 2000); (b) production of the plant growth hormone indole-3-acetic acid; (c) relative harmlessness to human health and the environment; and (d) low level of antibiotic resistance (Berg, 2000). The objective of our study was to evaluate *S. plymuthica* strain HRO-C48 for biocontrol of Verticillium wilt and Phytophthora root rot in strawberry. During three consecutive years, the efficacy of this biocontrol strain in field trials on three different locations naturally infested by *Verticillium* or *Phytophthora* was analyzed. The field trials were integrated into commercial strawberry production.

Material and methods

A spontaneous mutant isolate of *S. plymuthica*, resistant to rifampicin HRO-C48*Rif* (100 µg/ml) was used in all experiments. No differences in colony morphology, antifungal properties, and growth rate were found between the mutant and wild-type. The strains of *S. plymuthica* were stored at -70°C in nutrient broth (nutrient broth 2 [6.75 g of peptone, 5 g of NaCl, 1.5 g of yeast extract, 1.75 g hydrolyzed protein in 1 liter of distilled water, 30°C, pH 7.2], Sifin, Berlin, Germany) containing 15% glycerol. The isolate was deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) as DSMZ 12502.

Field trials I (1997, at Stuthof) and II (1998, at Goorstorf) were conducted in Mecklenburg-Western-Pomerania, in areas naturally infested by *V. dahliae*. Soil population density of *V. dahliae* in Stuthof and Goorstorf was 40 and 21 microsclerotia/g soil. In 1999, field trial III was carried out in Bühl, Baden-Württemberg, in a field naturally infested by 1000 CFU/g soil *P. cactorum*. Soil parameters at all locations were analyzed by LUFA, Rostock or Bonn, Germany). In Stuthof (trial I), the soil texture was loamy sand, pH = 5.5, 1.7% organic matter and the following contents of nutrients (mg/100 g soil): P_2O_5 12, K_2O 15, Mg 6. In Goorstorf (trial II), the soil texture was sand, pH = 5.7, 1.3% organic matter and the following contents of nutrients (mg/100 g soil): P_2O_5 16, K_2O 8, Mg 11. In Bühl (trial III), the soil texture was sandy loam, pH = 5.1 and the following contents of nutrients (mg/100 g soil): P_2O_5 24, K_2O 33, Mg 5. In trials I and II, approximately 1,200 strawberry plants cv. Elsanta were grown in a completely randomized block design with six replicates. The strawberries were planted as frigo-plants in May, harvested as runner plants in autumn, and stored at -2°C during the winter. In trial III, approximately 100 strawberry plants cv. Elsanta per treatment were grown in a completely randomized block design with 4 replicates. The roots of strawberry plants were dipped in a bacterial suspension (4 x 10^9 CFU/ml) immediately prior to planting. Controls were dipped in tap water. In July the fruits were harvested and weighed to determine yield and % wilted and dead plants. In field trial III, the strawberries were planted as runner plants in August and treated with *S. plymuthica* as described above. Disease incidence based on a 0-2 scale (0 = no disease; 1 = infected plant showing wilting symptoms; and 2 = dead plants) was recorded in September and October of 1999. At four different sampling times, plant roots with adhering soil taken from five plants from one treatment were aseptically sampled into sterile Stomacher bags and treated as one sample. Five replicates for each treatment were investigated. Five g of each sample was extracted in a Stomacher laboratory blender (BagMixer) with sterile NaCl solution (8.5 g/l). Solutions were serially diluted and plated on nutrient agar 2 containing 100 ppm rifampicin (Fluka); CFUs were determined after five days incubation at 20°C.

Fingerprinting of the bacterial rhizosphere communities by DGGE was carried out as described previously (Heuer & Smalla, 1997). Briefly, 16S rDNA fragments (positions 968 to 1401 [*Escherichia coli* rDNA sequence]) were amplified by PCR from DNA extracts with the primer pair F984GC-R1378. The amplicons were separated at 60°C in a denaturing gradient of 40% to 58% of 7 M urea and 40% (v/v) formamide. Acid silver staining was used for the detection of DNA in DGGE gels. Differences between the treatments in the biocontrol experiments were determined by U-Test "Mann-Whitney" ($P \leq 0.05$) by Statistical Product and Service Solutions for Windows, Rel. 9. 0. 1. (SSPS Inc., Chicago, Illinois).

Results

Effect of* S. plymuthica *HRO-C48 on disease incidence and strawberry growth yield in field

Three field trials at different locations were carried out to evaluate the ability of *S. plymuthica* HRO-C48 to suppress the pathogens under natural conditions. In field trial I (1997, Stuthof),

wilting symptoms of *V. dahliae* were reduced (0 – 37.7%) in plots treated with strain *S. plymuthica* (92 diseased plants/235 healthy plants) compared with nontreated control plots (203 diseased plants/190 healthy plants). In field trial II (1998, Goorstorf), symptoms of *V. dahliae* did not develop. Differences in disease incidence between the control and *S. plymuthica* HRO-C48-treated plants were not detected. In field trial III, the reduction of Phytophthora root rot by the treatment with *S. plymuthica* compared with the nontreated control ranged from 1.3 to 17.9%, with an average of 9.6%. In field trial I (1997), the yield of strawberries in plants treated with *S. plymuthica* (32.5 kg per 100 plants) was 296% greater than the nontreated control (8.2 kg per 100 plants). In field trial II (1998, Goorstorf), yield was not different between the control and plants treated with *S. plymuthica*. In field trial III, plants treated with *S. plymuthica* exhibited greater growth in September, one month after planting in September and in October. *S. plymuthica* HRO-C48 was re-isolated throughout the 14-month period. In field trial I, *S. plymuthica* were recovered at the second (flowering plants) and third (fruitioning plants) sampling periods. For both field trials I (1997, Stuthof) and II (1998, Goorstorf), *S. plymuthica* HRO-C48*Rif* decreased each period. Fourteen months after planting, *S. plymuthica* was detected at significantly lower levels $\log_{10}$ 2.9 CFU/g root in Stuthof in 1998 (field trial I). In 1999, two years after inoculation, the strain was not recovered from the soil in Goorstorf (field trial II).

Environmental factors

Weather conditions during the three field trials I (1997), II (1998), and III (1999) were different. The vegetation period in 1997, started with a cold and rainy spring (May: temperature = 10.8°C, rainfall = 2.4 mm) during planting. This period was followed by a warm, dry summer with a maximum temperature of 21°C in August and low rainfall (1.9 mm) during the flowering period and harvest. In contrast, the spring of 1998, was not as cold as the spring of 1997 (May = 12.9°C) and the total rainfall level was lower (1.4 mm). In 1998, during the flowering period and harvest, the temperature between June and October was generally lower (13.9°C on average) compared with 1997 (15.4°C). The amount of rainfall from June to October was higher than the year before. After harvest at the end of October, the conditions in both years (1997/1998) were similar with an average temperature of 8.7°C. The level of rainfall per month ranged from 1.4-1.9 mm. The third trial located in Bühl had higher average temperatures per month and rainfall than the other trails.

DGGE analysis of thebacterial rhizosphere community

Total DNA was extracted from selected rhizosphere samples. A fragment of the 16S rRNA gene was amplified by PCR and separated in DGGE according to sequence-dependent melting differences. For each sampling, the DGGE fingerprints were compared between inoculated and uninoculated roots. HRO-C48 was not detectable in any DGGE fingerprint. No differences became apparent between DGGE fingerprints of different treated roots, both showing the same evident seasonal shifts.

Discussion

A requirement for an efficient biocontrol agent is the ability to survive and to become established in the rhizosphere (Lugtenberg et al., 2001). The rhizosphere competence of *S. plymuthica* HRO-C48 was demonstrated by re-isolation of the rifampicin-resistant mutant from the rhizosphere at levels of approximately 3 to 7 $\log_{10}$ CFU/g root over a period of 14 months under field conditions.

In our study, a high biocontrol activity was correlated with high yield enhancement. Increases in yield were not observed when disease symptoms were absent, e.g., in field trial II (1998, Goorstorf). In field trial I (1997, Stuthof) and III (1999, Bühl) disease incidence was

high and *S. plymuthica* HRO-C48 reduced disease symptoms and to enhance the yield. In general, the incidence of Verticillium wilt is influenced by the initial inoculum density of microsclerotia, the cropping history of the field site, the a-biotic conditions such as weather conditions and stress, soil parameter and the presence of other pathogens, such as nematodes (Green, 1980; Harris and Yang 1996). In our study, different levels of microsclerotia in the soils of the field trials occurred. In field trial I the number of microsclerotia was higher compared to field trial II. Only in field trial I, symptoms of *V. dahliae* were monitored. However, mean values from 1-2 microsclerotia/g soil of the fungus have been demonstrated to infect the susceptible strawberry cultivar Elsanta under optimal conditions for an infection of the fungus (Harris & Yang, 1996). The cropping history of a site is also a good indicator of wilt risk. Plants that increase the soil inoculum of *V. dahliae,* such as potato, cotton, brassica crops, sugar beet and miscellaneous vegetables enhance the potential risk of infection. Field trial I was planted after oilseed rape, a host of *V. dahliae* which was responsible for the high inoculum level of *V. dahliae*. In contrast, field trial II was planted on a former barley field, a non-host plant of *Verticillium*. Weather conditions also had an influence on the field trials. For example, the infection data of field trial I reflect the fact that the years had a warm, dry summer that are known to favor wilt in strawberry. Soil parameters in the different trials were very similar and we do not consider them a factor.

The development of a commercial formulation of DSMZ 12502 is in progress, and in the near future RhizoStar® will be commercially available.

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Pathogen defence against biocontrol

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Abstract: Antibiotics produced by antagonistic bacteria play a key role in biocontrol of plant pathogenic fungi. Under field conditions, however, the efficacy of antibiotic-producing bacteria to control plant pathogens has been inconsistent. This variability has been attributed to multiple factors, including inefficient root colonisation by the introduced bacterial strain, and temporal and spatial variation in the expression of antibiotic biosynthetic genes. Most of the factors studied with respect to improving the efficacy of biocontrol relate to improving the behaviour and activity of the biocontrol agent itself. In contrast, very little attention has been paid to the target pathogen and, more specifically, to mechanisms by which the pathogen can defend itself against biocontrol agents. It is generally assumed that resistance to antibiotics produced by biocontrol agents only occurs at a very low frequency, because (1) most biocontrol bacteria produce multiple antibiotics, (2) the antibiotics are produced in microsites and (3) produced during a short period in the life cycle of a pathogen. Several studies, however, have shown that there is considerable variation in sensitivity among pathogenic fungi to antibiotics produced by antagonistic bacteria. Furthermore, we identified fungal isolates from the field that were not only insensitive to one but two bacterial antibiotics, indicating that resistance to multiple, chemically unrelated, antibiotics may exist. In this study, we investigated the variation in sensitivity of several pathogenic fungi to antibiotics produced by *Pseudomonas* spp. The nature of the defence mechanisms involved and the implications for the efficacy and implementation of biocontrol agents are discussed.

Introduction

History has demonstrated that plant pathogenic organisms can have a devastating effect on crop yield. Next to food shortages, plant pathogens, like fungi, can produce toxins and can therefore seriously affect human and animal health. For these reasons, it has always been of great importance to reduce crop losses and to suppress the development of plant pathogens. The suppression of plant diseases will become increasingly important again since the success and widespread use of many pesticides is rapidly declining. Resistance development in plant pathogens and the hazardous side effects of many of these pesticides make many of these compounds inappropriate for further application. Without adequate alternative strategies to suppress the development of plant pathogens it is inevitable that the prevention of plant diseases will become increasingly difficult.

Biocontrol of plant pathogens by antagonistic bacteria has caught considerable attention since it not only can replace the use of hazardous chemical pesticides but also may provide control of diseases that cannot, or only partially, be managed by other control strategies (Cook, 1993). Although initially not considered commercially feasible, extensive research performed resulted in the development and marketing of biocontrol agents as commercial products.

Biocontrol of plant pathogenic fungi by bacteria

The success of biocontrol of soil borne fungi is the outcome of complex interactions between bacteria, the fungus and the host plant, which can be further complicated by a-biotic factors, like temperature, pH and chemical composition of the soil. A key factor to efficiently control plant pathogens is the ecological rhizosphere competence of the antagonistic bacterium. A bacterium must be an efficient root colonizer and capable of defending its habitat and out-competing the plant pathogen. In the broad sense, any trait that increases the ecological rhizosphere competence can add to the successful control of plant pathogenic fungi.

It was demonstrated that the production of antibiotics by bacteria is an important trait for the ecological rhizosphere competence. Soil-borne bacteria produce a vast array of different antibiotics (Whipps, 2001). Some of these compounds are related, like the phenazines, while others belong to entire different classes and are synthesized through entirely different enzymatic pathways. The elucidated or suggested modes of action of these antibiotics also show a great variety, ranging from disruption of membranes by surface active antibiotics, like rhamnolipids (Jarvis & Johnson, 1949; Ochsner et al., 1994), to interference with replication by intercalation into the DNA, and indirectly increasing the intracellular accumulation of active oxygen species (AOS), such as superoxide, hydrogen peroxide and hydroxyl radicals.

Bacterial antibiotics have several advantageous features, which make them particularly interesting. First, in many cases these anti-fungal compounds do have, next to broad spectrum anti-fungal (*Gaeumannomyces, Thielaviopsis, Fusarium, Septoria, Rhizoctonia*) properties, also anti-oomycotic (*Pythium*), and anti-helminthic (*Globodera*) properties (Keel et al., 1992; Thomashow & Weller, 1996, Cronin et al. 1997). Second, the antibiotics can be purified and applied independently of the synthesizing bacteria in bio-control of seed-borne diseases (Kerr, 1980, Stabb et al., 1994, Millner et al. 1996). Third, these compounds can be interesting 'lead'-compounds for the development of more potent fungicides, which are environmentally less hazardous when compared the currently used fungicides.

Inconsistencies in biocontrol under field conditions

Biocontrol of plant pathogenic fungi under controlled conditions by means of antibiotics or antibiotic producing bacteria has shown promising results. However, under regular field conditions this approach shows inconsistencies (Weller, 1988). These are mostly attributed to insufficient root colonizing abilities or temporal and spatial variation in antibiotic production or activity. A-biotic factors like oxygen, temperature, specific carbon and nitrogen sources, and micro-elements have been identified or proposed to influence the *in situ* antibiotic production by fluorescent *Pseudomonas* spp. (reviewed by Duffy & Defago, 1997). The toxicity of antibiotics can be significantly influenced by the pH, like has been found for phenazines (Chin-A-Woeng et al., 1998) and 2,4-diacetylphloroglucinol (De Souza et al., unpublished data). Biotic factors, like the plant (Georgakopoulos, et al., 1994; Maurhofer et al., 1995), the pathogen (Duffy & Defago, 1997), the indigenous microflora (Wood et al., 1997) and the cell density of the antibiotic producing strain (Wood & Pierson, 1996; Raaijmakers, et al., 1999) also have a determinative role in antibiotic production.

Variation in sensitivity of fungi to antibiotics produced by biocontrol agents

When considering the reported inconsistencies in biocontrol little attention is paid to the target plant pathogens and the possibility that these develop resistance against the antibiotics. Since most of the bio-control agents produce more than one antibiotic it is generally assumed that

resistance to multiple antibiotics only occurs at a very low frequency. Furthermore, the selection pressure is considered low since the antibiotics are present in microsites on the roots and therefore the pathogen population is not entirely exposed (Handelsman & Stabb, 1996).

Nevertheless, there are several indications that plant pathogenic fungi and oomycetes can have a significant influence on the success of biocontrol. *Pythium* species (oomycete) showed considerable variation in sensitivity towards phenazine-1-carboxilic acid (PCA) (Gurusiddaiah et al., 1986) and Rhizoctonia solani anastomosis groups varied in sensitivity for gliotoxin (Jones & Pettit, 1987). Mazzola et al. (1995) characterized natural *Gaeumannomyces graminis* strains that varied with respect to sensitivity for phenazine-1-carboxylic acid (PCA) and 2,4 diacetylphloglucinol (DAPG). Moreover, some of these strains were less sensitive for both PCA and DAPG, demonstrating that resistance to unrelated antibiotics may exists. Their presence can have a significant negative impact on the efficacy of biocontrol rhizobacteria that produce these antibiotics and durability of biocontrol.

Fungal resistance against anti-fungal compounds

Although in the area of biocontrol very little is known about resistance development and the mechanisms on which this resistance is based, there are some significant leads from other research areas. Plants also produce anti-fungal compounds, known as phytoanticipins and phytoalexins, and various resistance mechanisms in fungi against several of those have been described (reviewed by Morrissey & Osbourn, 1999). In many cases resistance is based on metabolization of the antibiotic. Saponins, like avenacin A-1 and α-tomatine, are cleaved by glycosyl hydrolases, resulting in less toxic products. Different pathogens, like *Botrytis cinerea*, *Septoria lycopersisci* and *Fusarium oxysporum* f.sp. *lycopersici*, produce different glycosyl hydrolases, all cleaving α-tomatine differently but all with the same detoxifying result. This indicates that all three resistance mechanisms have evolved independently. Other described enzymes capable of degrading different phytoanticipins and phytoalexins are hydratases and cytochrome P450-dependent monooxygenases. Especially the latter enzymes are notorious with respect to detoxification of all kinds of xenobiotics, leading to resistance not only in fungi, but also insects (reviewed by Scott, 1999). Cytochrome P450-dependent monooxygenases can have a wide variation in substrate specificity and can produce all kinds of products by a vast array of reaction mechanisms (Mansuy, 1998). Remarkably, monooxygenase-mediated detoxification has the potential to confer cross-resistance to toxins independent of their target sites (Oppenoorth, 1985; Scott, 1991; Wilkinson, 1983).

Next to degradation, transporter proteins, situated in cell membranes can also contribute to antibiotic resistance. By transporting toxic compounds out of the cell, the organism can contain the damage that these compounds can inflict. These efflux pumps can be divided into two major groups, the ATP-binding cassettes (ABC) and major facilitator superfamily (MFS). ABC transporters comprise the largest number of efflux pumps and account for transport of both endogenous and exogenous toxicants (Higgins, 1992). Recently, it was demonstrated that one ABC transporter in *B. cinerea*, *BcatrB*, plays a role in the sensitivity towards the fungicide fenpiclonil, the phytoalexin resveratrol and the bacterial antibiotic phenazine (Schoonbeek et al., 2001 and unpublished data). This emphazises the fact that ABC transporters, similar to cytochrome P450-dependent monooxygenases, can be involved in cross-resistance to unrelated chemicals. Collectively, these examples demonstrate that resistance to antibiotics is not hypothetical and indeed do occur. It is inevitable that development of biocontrol systems must deal with potential defense mechanisms in the target pathogen to design durable crop protection measures. By understanding the biochemical and

genetic basis of the involved resistance mechanisms, innovating strategies can be devised to improve biocontrol.

Variation in sensitivity of Fusarium oxysporum to 2,4 diacetylphloglucinol (DAPG)

A collection of natural *Fusarium oxysporum* isolates was screened for sensitivity to 2,4 diacetylphloglucinol (DAPG). This collection contained 96 isolates from different formae speciales and isolates that are classified as non-pathogenic. In *in vitro* experiments the radial growth of each isolate was monitored during growth on plates containing different DAPG concentrations. For approximately 90% of these isolates growth stopped at concentrations between 25 and 50 µg/ml DAPG. However, several isolates were still capable of growing at concentrations of 400 µg/ml. DAPG extraction followed by HPLC analysis revealed that for some of these insensitive isolates the DAPG had disappeared underneath the growing mycelium. Apparently, DAPG is degraded and thus detoxified by some insensitive isolates. Since no degradation products could be identified yet, the mechanism by which DAPG is degraded is still unclear. Next to degradation, also other mechanisms may play a role since some insensitive isolates apparently do not degrade DAPG. Based on these initial results it can be concluded that *F. oxysporum* isolates collected from the field can vary significantly in their sensitivity to the antibiotic DAPG and that different mechanisms are responsible for insensitivity.

Collectively, our results and the examples from literature demonstrate that resistance of plant pathogenic fungi to antibiotics is not hypothetical and indeed does occur. It is inevitable that development of biocontrol systems must deal with potential defense mechanisms in the target pathogen to design durable crop protection measures. By understanding the biochemical and genetic basis of the resistance mechanisms involved. Innovating strategies can be devised to improve biocontrol.

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Impact of *Fusarium* and DON-production on expression of two chitinase genes of *Trichoderma atroviride* P1

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Abstract: Fusarium head blight of wheat caused by mycotoxigenic *Fusarium* is an increasing threat worldwide to crop, animal and human health. The fungi survive and sporulate in crop residues. *Trichoderma atroviride* is a potential biocontrol agent for *Fusarium*. We tested the impact deoxynivalenol (DON) producing *F. graminearum* and *F. culmorum* strains on expression of two *Trichoderma* chitinase genes, *ech42* and *nag1*, which are important for biocontrol activity. *Trichoderma* gene expression was monitored in assays in vitro and on maize crop residues using *goxA* reporter gene fusions. We found that DON-producing *Fusarium* strains repressed expression of *nag1* but not *ech42*. DON-negative *Fusarium* or a non-producing *Tri5* mutant had no effect on biocontrol gene expression. Synthetic DON added in assays with non-producing Fusarium resulted in repression of *nag1*. This is the first demonstration of a target pathogen down-regulating genes in a fungal biocontrol agent, and suggests a novel ecological function for mycotoxins as a factor in *Fusarium* competitiveness.

Key words: *Trichoderma atroviride* P1, *Fusarium graminearum*, *Fusarium culmorum*, Deoxynivalenol, gene expression, mycotoxin

Introduction

Fusarium graminearum and *F. culmorum* cause Fusarium head blight; an important disease on a wide range of plant species, including many important crop plants. Infections reduce both yield and quality of the grain. The reduction in quality is partially due to a number of mycotoxins, including the trichothecene deoxynivalenol (DON) produced by these fungi. *F. graminearum* and *F. culmorum* survive and sporulate in crop residues; the major sources of inoculum for the following crops. In animals and human DON causes feed refusal, vomiting and is immunosuppressive. The antagonistic fungus *Trichoderma atroviride* P1 is a potential biocontrol agent against a wide range of aerial and soilborne plant pathogens. Its mycoparasitic activity may be due the production of several chitinolytic enzymes such as the ECH 42 endochitinase and an N-acetyl-β-glucosaminidase (NAG1) that are involved in mycoparsitism. The *Trichoderma* strains used contain a fusion of the *Aspergillus niger goxA* reporter gene with either the *ech42* or *nag1* 5' upstream noncoding sequences (Mach et al., 1999).

Material and methods

Fungal strains and culture conditions

T. atroviride strain P1 (formerly *T. harzianum*) and derivatives carrying reporter gene fusions to *ech42* and *nag1* chitinase biosynthetic genes used in this study have been previously described (Mach et al., 1999). *F. culmorum* strains 9712 (F.c. 9712) and 9713 (F.c. 9713)

were field isolates. *F. graminearum* field isolate GZ3639 (F.g. GZ3639) was isolated from scabby wheat, strain *F. graminearum* GZT40 (F.g. GZT40) contain the transformation vector pGZTS4-1 which disrupt the Tri5 gene and produce no DON (Proctor et al., 1995).

Influence of different media on growth and chitinase gene expression in* T. atroviride *P1

One plug of an actively growing culture of *T. atroviride* derivatives containing either the *ech42-gox* or the *nag1-gox* fusion was inoculated on four different media. 1.5, respectively, 0.1 % malt agar, 1/5 PDA (4.8g Potato Dextrose Booth, 12g agar per liter, pH 6.5), SM (2.8 g $(NH_4)_2SO_4$), 0.6 g urea, 4.0 g KH_2PO_4, 0.6 g $CaCl_2*2H_2O$, 0.2 g $MgSO_4*7H_2O$, 0.01 g $FeSO_4*7H_2O$, 0.0028 g $ZnSO_4*2H_2O$, 0.0032 g $CoCl_2*6H_2O$, 12 g agar per liter, pH 5.4)). *T. atroviride* P1 was grown alone or co-inoculated with the non DON-producing *F. graminearum* strain GZT40 in distance of 3.2 mm for 66h in the dark. Glucose oxidase activity was measured as described above.

***Influence of synthetic DON on* Trichoderma *gene expression* in vitro**

200 µl of a sterile DON-solution (0, 1, 2.5, and 5 ppm, respectively) were platted on a 1.5 % malt agar plate. The plates were inoculated with one plug of an actively growing culture of *T. atroviride* P1 derivative ech42 or nag1. Glucose oxidase activity was determined after 66 hrs of growth as described below. Means of three experiments with six replicate plates were separated using Fisher's protected ($P \leq 0.05$) least significant difference (LSD) test.

***Influence of different* F. culmorum *and* F. graminearum *strains on* Trichoderma *chitinase gene expression* in vitro**

One plug of an actively growing culture of F.c. 9712, F.c. 9713, F.g. GZ3639 or F.g. GZT40 was inoculated on 1.5 % malt agar plates. These plates were co-inoculated after 24 hrs with one plug of an actively growing culture of *T. atroviride* P1 derivatives ech42 or nag1 respectively in distance of 3.2 cm of the *Fusarium* plug. Glucose oxidase activity was determined after 66 hrs of growth as described below. Means of three experiments with six replicate plates were separated using Fisher's protected ($P \leq 0.05$) least significant difference (LSD) test.

Measurment of glucose oxidase activity

10 ml of a phosphate-puffer was added to the plates. After rotary shaking for 30 min at 150 rpm, the liquid phase was transferred in a 15 ml falcon tube and centrifuged for 5 min at 3200 rpm. The resulting supernatant was used for quantification of glucose oxidase activity (Mach et al., 1999)

Influence of different* F. culmorum *and* F. graminearum *strains on* Trichoderma *chitinase gene expression on crop residues of the two maize varieties Magister and Corso

Leavess of the maize varieties Magister and Corso were cut into pieces and surface sterilized. Flasks containing 5 g of plant material were inoculated with two plugs of *Fusarium* strains F.c. 9712, F.c. 9713, F.g. GZ3639 or F.g. GZT40 and two plugs of *T. atroviride* P1 derivatives *ech42* or *nag1*. After one week of growth, 20 ml of phosphate puffer was added to each flask. After rotatry shaking for 10 min at 150 rpm, the liquid phase was transferred into a 50 ml falcon tube and centrifuged for five min at 3200 rpm. The resultant supernatant was analyzed for glucose oxidase activity according to Mach et al. (1999). Means of three experiments with eight replicate plates were separated using Fisher's protected ($P \leq 0.05$) least significant difference (LSD) test.

Results

Different media alter expression of key biocontrol factors in* T. atroviride *P1

The growth of both *Trichoderma* derivatives was altered by different media (data not shown). In order to avoid effects of fungal growth caused by different media, glucose oxidase activity is always reported per growing area. Expression of *nag1* and *ech42* was similar. Expression of both chitinase genes differed significantly on different media. Highest expression per growing area was found, when grown on 1.5 % malt agar plates, lowest on 0.1 % malt media, indicating that both biosynthetic genes are inducible. Expression on PDA and SM was on an intermediate level. Co-inoculation with the low DON producing strain 9712 had no significant influence in all treatments with exception on SM media for *nag1* expression, which was induced in the presence of *Fusarium* (Figures 1 & 2).

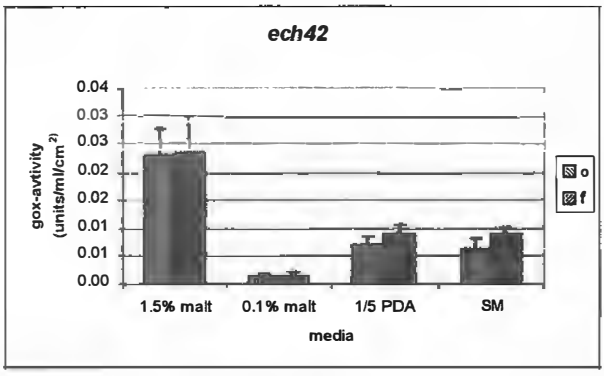

Figure 1. Expression of the *ech42-gox* fusion in *T. atroviride* P1 after 66 h of growth at 24°C in the dark on four different media. Values are expressed as units per ml extraction puffer and growing area. *Trichoderma* was growing alone (o) or co-inoculated with the non DON producing *Fusarium graminearum* strain GZT40 (f). Each value is the mean of three experiments with six replicate plates. Error bars represent the SD of the means.

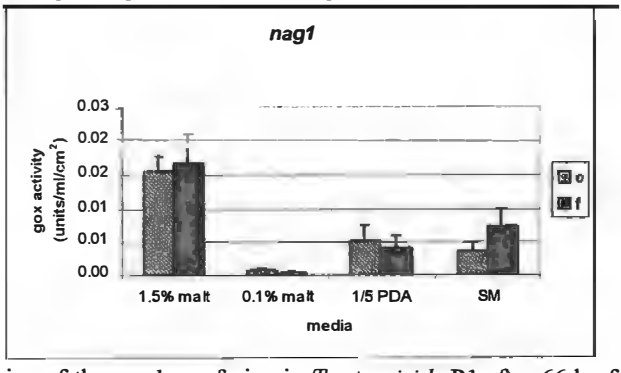

Figure 2. Expression of the *nag1-gox* fusion in *T. atroviride* P1 after 66 h of growth at 24°C in the dark on four different media. Values are expressed as units per ml extraction puffer and growing area. *Trichoderma* was growing alone (o) or co-inoculated with the non DON producing *Fusarium graminearum* strain GZT40 (f). Each value is the mean of three experiments with six replicate plates. Error bars represent the standard deviations of the means.

***Influence of synthetic DON on* Trichoderma *gene expression* in vitro**
Addition of 200 µl of synthetic DON to the media repressed expression of *nag1*, but not *ech42*.

***Influence of different* F. culmorum *and* F. graminearum *strains on* Trichoderma *chitinase gene expression* in vitro**
The presence of different *Fusarium* strains co-inoculated with *T. atroviride* P1 affected *nag1-gox* gene expression to different extents. The DON producing wild type *Fusarium* strains F.c. 9712 and F.g. GZ3639 repressed the expression of the *nag1* gene by 23% and 39%, respectively compared to the control without *Fusarium*. The low-DON producing wild type strain F.c. 9713 and the DON-negativ mutant F.g. GZT40 did not affect *nag-gox* reporter gene expression. The *Fusarium* strains used had no influence on glucose oxidase activity of *ech42*.

Influence of different* F. culmorum *and* F. graminearum *strains on* Trichoderma *chitinase gene expression on crop residues of the two maize varieties
The DON producing wild type strains F.c. 9712 and F.g. GZ3639 repressed the expression of *nag1* gene by 44% and 49 %, respectively on crop residues of maize variety Corso and by 24% and 20% on residues of maize variety Magister compared to the control without *Fusarium*. The low-DON producing wild type strain F.c. 9713 and the DON-negativ mutant F.g. GZT40 did not affected *nag-gox* reporter gene expression on crop residues of both maize varieties Corso and Magister. The *Fusarium* strains used had no influence on glucose oxidase activity of *ech42*.

Discussion

Addition of synthetic DON and the presence of DON-producing Fusarium strains in vitro and on residues of maize residues repressed expression of the *nag1-gox* fusion but not *ech42* expression. These results indicate that the mycotoxin deoxynivalenol inhibits *nag1* expression. We suggest that DON increase the competitiveness of Fusarium by inhibiting important biocontrol factors of antagonists.

Acknowledgements

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General discussion

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The reality - a commercial perspective to plant disease biocontrol

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Abstract: In spite of the fact that a number of microbial preparations have been developed and are starting to penetrate the market, control of plant diseases is still largely carried out by chemicals and cultural practices. As their mode of action is targeted to bacteria and fungi, the compatibility of many of them with beneficial insects and mites is generally satisfactory and there has been no real need to develop biological products for disease control. Nor has there been significant pressure from the market to develop alternatives. Recent changes in the regulatory climate may stimulate some uptake of alternative products, however. The world market for pesticides is about $ 30 billion and of this less than 1% is biopesticides, which is about $ 300 million. This includes all non-synthetic chemicals, such as microbial pesticides, beneficial arthropods, natural pesticides and pheromones. In microbials, most of this is accounted for by *Bacillus thuringiensis* The global biopesticide (real microbials) market is estimated at $ 160 million in 2000 - over 90% of this was accounted for by B.t.'s. This leads to about $ 16 million for microbial pesticides worldwide, excluding B.t. sales. The development and success of bio products will be highlighted with regard to the market; the biopesticide industry and governmental factors with emphasis on registration. The status and commercial future of microbial products as seen from a commercial point of view will be discussed. The potential for these products to be developed and marketed will be considered in light of market incentives, costs for development and registration and governmental policy. Finally, the question of whether there can be any real progress expected in biocontrol of diseases will be addressed.

Key words: biological control, biopesticides, commercialisation, disease, market, registration

Introduction

Despite a great deal of research on biocontrol of diseases relatively few products for managing diseases have been developed, registered and successfully marketed for biocontrol of plant diseases. Biocontrol of insects and mites, on the other hand, has enjoyed more than thirty years of success. Chemical fungicides have been used to treat diseases, soil-borne and foliar, for much of the twentieth century and still dominate the market today. There have been no significant pressure from the market to develop alternatives (Elad, 1990). Recent changes in the regulatory climate may stimulate some uptake of alternative products, however. The working group of Biocontrol of Fungal and Bacterial Plant Pathogens at the IOBC WPRS was established in 1991 in order to promote the implementation of biocontrol of plant diseases. During the decade of activity the subject was followed and discussed and it is evident that in the recent years more and more commercial products are presented and followed either on a research level or on commercial basis (Elad et al., 2001). The potential for biocontrol products will be considered in light of market incentives and governmental policy. Finally, the question of whether there can be any real progress expected in biocontrol of diseases will be addressed. For an overview of biocontrol of diseases from a research perspective, see Paulitz & Bélanger (2001) and Elad & Freeman (2002).

Microbial disease control products

Only a small number of products have actually been registered and are commercially used by growers. Below the most important microbial disease control products are given with some specific information on their importance and registration status. More information on microbial pesticides is given by Butt et al. (1999), Fravel et al. (1999) and Paulitz & Bélanger (2001).

AQ10 (Ecogen Inc, USA) based on *Ampelomyces quisqualis* (strain M-10) and sold as a biofungicide for the control of powdery mildews. It is used in grapes, and many other crops, including greenhouse crops. It is registered in the USA and pending for inclusion in Annex I in Europe and provisionally registered in Italy. The use in protected crops is still restricted.

Binab (Binab Bio-Innovation AB, Sweden) based on *Trichoderma harzianum* and *T. polysporum* and sold as a biofungicide for control of diseases in strawberries and greenhouse crops: tomatoes, cucumbers and ornamentals. It was the first biofungicide registered in a European country, *i.e.* in France in 1976 for wounds on trees. In 1985 it was registered in Sweden for use on greenhouse crops, followed by Denmark. It is aimed at soil-borne fungal diseases in greenhouse vegetables, ornamentals and other crops and *Botrytis* in strawberries. In this crop bumblebees are used to vector the antagonists to the flowers. There are four formulations that are sold as a plant growth enhancer, a biofungicide and a soil amendment.

Contans (Prophyta GmbH, Germany) is based on *Coniothyrium minitans* and sold as a biofungicide for control of *Sclerotinia sclerotiorum*, *S. minor* and *S. trifoliorum*, in high value agricultural and horticultural crops. In greenhouses it can be used in lettuce, celery, beans, tomatoes, cucumbers and ornamentals. The registration is pending in the EU as a new active ingredient, and it has been sold in Germany on a preliminary registration since 1997 for lettuce in greenhouses. Registration is expected soon in some other EU countries once the A.I. will be placed on Annex I. It is registered in Hungary, Poland, Switzerland and the USA.

Mycostop (Kemira Agro Oy, since 2002 called Verdera Oy, Finland) it has been on the market since 1990 and is available in the greatest number of countries. This product is based on *Streptomyces griseoviridis* (K61) and sold for control of damping-off, root and stem rot diseases in greenhouse cucumbers, tomatoes, peppers, lettuce, and ornamentals. While it mainly targets *Fusarium*, it also controls or suppresses *Pythium*, *Phomopsis*, *Rhizoctonia*, *Phytophthora*, *Botrytis*. It can be used as a seed treatment, on seedlings and in the crop itself. It is registered in Bulgaria, Canada, Denmark, Estonia, Finland, Hungary, Iceland, Italy, Latvia, the Netherlands, Norway, Russia, Spain, Sweden, Switzerland and USA.

Plantshield /Rootshield (Bioworks Inc., USA) is based on *T. harzianum* (T22) and sold as a biofungicide in the USA for control of damping-off diseases and root diseases in greenhouse and other crops for control of *Fusarium*, *Rhizoctonia*, *Pythium* and others. It is registered in the USA in Canada. There are two formulations, a wettable powder and a granulate, to be mixed with the growing medium. The same product is called *Trianum* in Europe, and will be distributed by Koppert BV as a plant strengthener in greenhouse vegetables and ornamentals. Registration is pending in a number of European countries. It is available now in Spain, UK and Norway and has been sold previously in some other countries as TRI 002 and 003.

Polyversum (Biopreparaty, Czech Republic) is based on *Pythium oligandrum* and sold as a defence inducer against diseases in crops such as grapes, wheat, hops and vegetables. It is also a plant growth promoter. It has been available for six years in a number of European countries, but its registration status is unclear.

Prestop (Verdera Oy, formerly Kemira Agro, Finland) is based on *Gliocladium catenulatum* (J1446) and is sold as a biofungicide for the control of *Pythium*, *Rhizoctoria*,

Phytophthora and for foliar diseases, such as *Didymella bryoniae* in cucumber. It has been registered in the USA since 1999 and is pending EU Annex I inclusion as a new active ingredient. It is already sold in Finland since 2001 based on a preliminary approval. This product will replace Gliomix.

Serenade (AgraQuest, USA) is based on *Bacillus subtilis* (QST 713) and sold as a biofungicide for control of many soil-borne and foliar fungal and bacterial diseases in field and protected crops. It is a preventative product that needs to be applied as a foliar spray. It is registered in the USA and in the EU the active ingredient is pending inclusion in Annex I.

SoilGard (Certis USA) is based on *Gliocladium virens* and sold for control of *Pythium*, *Rhizoctonia* and *Fusarium*. It is registered in the USA.

Sporodex (Plant Products LTD., Canada) based on *Pseudozyma flocculosa* (syn. *Sporothrix flocculosa*) and is aimed at powdery mildews of various crops.

Taegro (FZB Biotechnik GmbH, Germany, and distributed by Taensa Inc., USA and various Bayer subsidiaries in Europe) is based on *B. subtilis* (FZB 24) and sold as a plant growth enhancer, suppressing also *Rhizoctonia* and *Fusarium* on many crops, including greenhouse crops. It is sold in Germany and the USA (registered) as a plant strengthener and is pending registration in some other European countries.

TRICHODEX (Makhteshim Agan, Israel) is based on *T. harzianum* (T39) and sold as a biofungicide for control of *B. cinerea* in grapes and greenhouse crops. It is also reported to be active against *S. sclerotiorum* and some more diseases. It is registered in Denmark, Germany, Greece, Hungary, Israel, Italy, Romania, USA, several other countries in the Southern Hemisphere, and pending in the Netherlands.

Natural disease control products

They are used for control of diseases, and sometimes even against insects. These natural disease products are based on one of the following groups of components or on combination(s) of them: algae, enzymes / proteins, milk (extracts), minerals, mixtures of bacteria (including metabolites) and/or fungi, oils (mineral, vegetable or essential), plant or seed extracts, salts, soaps/fatty acids, sugars, sulphur and vitamins. Often research and registration approval may be lacking. Claims associated with these products can be: plant strengtheners, plant-growth promoter, soil-improver, induced resistance, etc. In the Netherlands over 150 products were found. Some of them, however, have been developed based on extensive research and could be useful products for disease control. As an example, *Milsana*, an extract from *Reynouria sachalinensis* was developed by the BBA in Darmstadt, Germany.

The biopesticide market and industry

The world market for pesticides is about $ 30 billion and of this less than 1% is biopesticides - about $ 300 million. This includes all non-synthetic chemicals, such as microbial pesticides, beneficial arthropods, natural pesticides and pheromones. Most microbial sales are accounted for by *Bacillus thuringiensis* for caterpillar and mosquito control. In Europe in 2000, sales of biopesticides including beneficials, microbial pesticides and pheromone products were $ 97 million - about 2% of the total European pesticide market. Sales of microbials were $ 25 million, representing 26% of the total biopesticide sales in Europe. Most of the biopesticides are used in countries that export vegetables, fruit and ornamentals. In the category of microbials also "soft" pesticides are included, such as fatty acids (Frost & Sullivan, 2001). The authors state that the new registration procedure in the EU has severely limited the availability of microbials, whereas B.t.'s were already available before 1993 and did not

suffer from this difficulty. They expect a yearly growth of 11.7 percent leading to about $ 210 million in 2007 for all biopesticides. The beneficials sector has attained a high percentage of saturation, so it is obvious that an increase of sales of microbials is expected. Jarvis (2001) estimates the global biopesticide (real microbials) market at $ 160 million in 2000 - over 90% of this are B.t.'s. This leads to about $ 16 million for microbials worldwide, excluding B.t. sales. If we compare the figures from Frost & Sullivan - $ 25 million for all microbials, including B.t., in Europe - with Jarvis - $ 16 million for all microbials world-wide excluding B.t. (ca 90%) - and estimate the non-B.t. microbials market for Europe (10-20%), this might be $ 2,5 - $ 5 million. It is a very small amount, and considering it is the total of ca 20-30 microbials (viral fungal and bacterial insecticides and fungicides one can see that the turnover per microbial is very low. Additionally, the sales are spread over sevearl countries for most products, giving small turnovers per country and relatively high marketing costs. After development and registration costs it is apparent that the biopesticide industry is small.

Jarvis (2001) estimated development costs for a microbial around $ 3 million, and if registration costs of at least $ 0,5 million are added, total costs are about $ 4 million. So, development costs of a product are around 10 – 20 times higher than a yearly turnover. Therefore, it is difficult to reach profitability on microbials. This is reflected by the failure of many companies who have started in this business. Lisansky (1997) mentioned that more than 175 have tried over the last twenty years but only few succeeded. This is partly due to faulty estimations on the development costs and time involved before a product will generate some return of investment, and partly due to a mistaken perception of the market. At the moment about 35 companies in Europe produce and/or sell microbial pesticides. Many of these are very small companies and provide local markets with their products. Often there is no registration approval for these products or the local legislation allows the sale under specific conditions. In the EU context, however, this is not allowed and with the notification of microbials in 2002, most of these products will either have to apply for registration or will no longer be allowed. Consolidation may be expected for companies involved in microbial pesticides, as we have seen in the agro-chemical industry.. This will indicate maturation of the industry as well as survival strategy and seems inevitable for the microbial industry.

Some microbials may do a better job if production costs are low, shelf-life is long, efficacy is good and the application simple. Microbials do have a number of drawbacks compared to chemicals, which are hard to overcome. These include a higher price, lower effectiveness, more complicated use, narrow spectrum and dependency on environmental conditions. B.t.'s and, to some extent, viruses come the closest to chemicals which may explain their success. Perhaps the market is not yet ready for microbials and only if real incentives develop will microbials be able to become profitable alternatives.

Registration and governmental policy

Registration procedures and costs are clear hurdles for the development of biological pesticides. Considering the market for a product and the development costs, as discussed above, it is obvious that registration costs are often too high to justify the investment. On top of this, procedures are not very clear and it is hard to estimate the costs involved up front.

In the EU, there are 17 micro-organisms registered as plant protection products. These were on the market before July 1993, the date of the enforcement of the EU Directive 91/414, and are considered as "old " active ingredients. "New" active ingredient will be registered through the EU Directive 91/414. The first one to be applied for was *Paecilomyces fumosoroseus* in 1994, even though the requirements for microbial pesticides only recently were established. *P. fumosoroseus* was recently included in the Annex I – the list of approved

new A.I.'s in the EU. Six others are waiting for inclusion also. In the USA there are 58 micro-organisms approved and the procedure here is much clearer and faster than in the EU which is indeed reflected in the number of approvals. In the EU almost every claim of disease control has to be proven by many official trials for each crop and for each disease. In the USA this is not required and this makes a registration much cheaper and faster.

As a consequence of the high registration costs we see many products on the market in the EU that are used illegally, without any registration. These are microbial as well as natural plant protection products. Some countries have a separate regulation in which products are more or less just notified and then approved for use on crops. In Germany about 170 products are on this list and widely used. Austria has something similar and in the Netherlands some products are also exempt of registration, although these are primarily used in the home and garden sector. Other EU countries have again other definitions in their pesticide legislation, which sometimes allow the sale of non-registered pesticides, depending on the claim. Also submission and evaluation costs differ enormously between countries. Recently the UK increased the fee for a microbial from £ 13,000 to £ 40,000. So even today within the EU, registration legislation is far from harmonised. There are attempts within the OECD (Organisation of Economic Co-operation and Development, The Pesticide Forum Working Group) to harmonise data requirements for microbials and pheromones. An EU attempt to set data requirements on a category "plant strengtheners" is on hold because of disagreements between countries. So we can conclude that registration is still a very difficult part of launching a microbial, despite all political rhetoric to stimulate the use of them.

Many governments are now and already for more than over ten years striving to reduce the use of chemicals. Success has been achieved in reducing insecticides and soil-disinfectants. In fungicides, no real reduction has been achieved. Only if governments develop tools to stimulate the development and registration of microbials might things change: for instance, lower and harmonised data requirements, lower submission and evaluation costs, faster procedures, and subsidies to stimulate companies taking risks in this field. Communication with the registration authority is often difficult, unless an application is submitted. The data on efficacy can be reduced, which will make a big difference in regard to costs. A microbial will perform in a certain environmental situation and/or climate and this may not have to be tested in each crop. We suggest testing major crops/sectors and extrapolating to a broader spectrum of application. In the USA no efficacy data are needed, but this leads to label recommendations that are not really supported by the product's ability. An in-between set of data seems a reasonable compromise for producers as well as regulatory bodies and will give users a proper idea on product performance.

Future perspective

Is the future really so bright for commercial microbial products? If the industry wants the products to succeed, they will have to be competitive with existing chemicals. As long as conventional fungicides are cheaper than microbial disease management products, they will continue to dominate the market for pesticides. Efficacy and user-friendliness must be improved if we are to move beyond the niche markets. There is a need to improve our marketing as well, and convince both the grower and governments that these products are desirable. Part of the larger problem is declining returns in agriculture. Growers need to receive better returns for their product if they are going to spend more capital on their inputs, such as more biological and natural solutions for their pest and disease problems.

It is unlikely that the success of beneficial arthropods will be followed by similar trends in microbial products. A much longer lead time and greater investments preclude this.

Moreover, the major producers of natural enemies are barely achieving profitability and development potential is limited. Also, many big players in the agro-chemical world have invested significant amounts of capital in this market and have largely withdrawn. The microbial pesticide industry is learning from the hard lessons of the past. Consolidation is foreseen which will hopefully lead to some long-lasting successful products and companies.

Collaboration between scientists is crucial in order to develop these products and contacts between these two groups are necessary from the outset of the development of a microbial. A good example is given by Paulitz & Belanger (2001) for the development of a powdery mildew control product, based on *Pseudozyma flocculosa*. In order to improve the prospects for implementation of biological disease control there is a need for intensive research in subjects that will assist the minimising the inconsistency and maximising the efficacy of microbial biocontrol agents. This includes the study of integration of biocontrol agents among themselves as was done for biocontrol of *B. cinerea* (Guetsky et al., 2001), taking into account the effect of environmental factors on the activity of biocontrol agents and integration with other control strategies. It is likely that in the future we will face microbial products with longer shelf life, better survival in the plant environment and higher efficacy. Much has also been learned looking at the chemical world regarding formulation and marketing of new products. Governments have a central role to play, in ensuring a level playing field in terms of registration. Scientists need to speak out forcibly against continuing cuts in research and development in the public sector. And industry has to improve its communication with all stakeholders and develop reliable microbial products. Real progress nevertheless, will only occur when registration is harmonised and the market demands non-synthetical chemical solutions for the production of food.

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Biocontrol of upper plant parts at pre and post harvest stages and involvement of microclimate

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Establishment of *Pantoea agglomerans* strain Eh 24 on pear blossoms and its compatibility with some chemicals

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Abstract: Fire blight caused by *Erwinia amylovora* has threatened the pear industry in Turkey since 1985. Use of epiphytic bacteria for biocontrol of *E. amylovora* has been considered as an ideal method to cope with the disease. In orchard trials conducted in 1999 and 2000 in Turkey, talc-based formulation of *Pantoea agglomerans* strain *Eh 24* reduced the percentage of blighted blossoms on pear orchards by 63.2 to 76.3%. For monitoring the colonization and population dynamic of the biocontrol agent, a strain of *P. agglomerans Eh 24* resistant to streptomycin (100 µg/ml) was sprayed on pear blossoms. It was determined that the population of *P. agglomerans* strain Eh-24 $^{strR+}$ on pear blossoms changed from $2x10^4$ to $1.3x10^6$ CFU per blossom within 18 days. The compatibility of *P. agglomerans* strain *Eh-24* with some chemicals, which are commonly sprayed on pear trees, was investigated in *in vitro* conditions. Copper hydroxide did not adversely affect the colonial development of strain *Eh-24* at the concentration as high as 100 µg/ml. Meanwhile, the colonial development of *E. amylovora* was rapidly decreased by 10 µg/ml Copper hydroxide. These results indicate that Copper hydroxide sprays can be used on pear trees as protective during winter treatment prior to antagonist application. We reached the stage of application for patent right of the bioformulation.

Key words: Biological control, fire blight, antagonistic bacteria, *Pantoea agglomerans* (*Erwinia herbicola*), *Erwinia amylovora*

Introduction

Fire blight, caused by *Erwinia amylovora*, has threatened the pear and apple industry in Turkey since 1985 (Öktem & Benlioğlu, 1988). Suppression of the blossom-blight phase of fire blight is the key in the management of this destructive and increasingly important disease (Schroth et al., 1974). Control of the disease is difficult since the effective bactericides, streptomycin and copper compounds, can not be routinely used in spray programs. Therefore, use of epiphytic bacteria for biocontrol of *E. amylovora* has been considered as an ideal method to cope with the disease (Beer et al., 1984). The Bacteria belonging to the species *Pantoea agglomerans* (*Erwinia herbicola*) and *Pseudomonas fluorescens* have been extensively tested as potential biocontrol agents of fire blight (Wilson & Lindow, 1993; Vanneste & Yu, 1996). Spraying apple and pear blossoms with suspensions of *P. agglomerans* suppressed fire blight in the same efficacy achieved by streptomycine (Wodzinski et al., 1990). Two orchard trials were conducted in 1999 and 2000 in the Aegean Region, turkey. In these trials, a talc-based formulation of *P. agglomerans* strain *Eh 24* was applied at 30 and 100% blooming; the biocontrol agent reduced the percentage of blighted blossoms by 63.2 to 76.3% (Özaktan et al., 2001).

The objective of this research were to monitor the colonization and population dynamic of biocontrol agent *P. agglomerans* strain *Eh 24* on pear blossoms under orchard conditions and to investigate the compatibility of this strain with some chemicals that are routinely used in pear orchards in *in vitro* conditions.

Material and methods

Antagonistic bacterium and its monitoring in pear blossoms

P. agglomerans strain *Eh 24*, which was previously isolated from pear blossom in İzmir province, and found effective against *E. amylovora*, was used as biocontrol agent in this study. Experiment was carried out in the pear orchard of the Faculty of Agriculture, University of Ege. For survival experiments, the strain *P. agglomerans Eh 24*, which is resistant to streptomycin (*Eh-24* $^{strR+}$), was used. Bacterial suspension (10^8 CFU/ml) was sprayed to runoff to marked branches of pear trees at the 20-30% and 70-90% bloom stage. The experiment was laid out in completely randomized block design with 3 replications (single trees). Antagonistic bacteria population was defined as follows: nine blossoms were sampled from each of the three trees and placed in sterile flasks. 0.1 M Phosphate Buffer was added to each flask and the flasks were shaken on a rotary shaker at 150 rpm for 10 min. Samples were diluted and sprayed on each of two Petri dishes containing SNA medium (saccharose nutrient broth agar), supplemented with streptomycin (100 µg/ml). blossoms were sampled for determining of *P. agglomerans* populations one, three, five, eight, ten, twelve and eighteen days after the second application (Stockwell et al., 1998).

In vitro *compatibility of P. agglomerans strain Eh 24 with some chemicals*

Mancozeb, Copper oxychloride+Maneb and Copper hydroxide are the most commonly used fungicides in pear orchards. In *in vitro* tests, these fungicides at various concentrations (3, 10, 30, and 100 µg/ml) were incorporated in SNA medium cooled to 40^0C. After pouring the medium was inoculated with 0.1 ml bacterial suspension (*P. agglomerans* strain *Eh 24*) of 10^6 CFU/ml. Evaluation of chemical activity was performed by accounting of colonial development of strain *Eh 24*. There were seven replications (Petri plates) for each chemical-dose. The chemicals which did not inhibit the colonial development of strain *Eh24* in the *in vitro* testes were tested for effectiveness against *E. amylovora* as described above.

Results and discussion

For monitoring the colonization and population dynamic of *P. agglomerans* strain *Eh 24*, pear trees were sprayed at 30% and full bloom with a suspension of cells of *Eh-24* $^{strR+}$. At the first sampling time, the bacterium was recovered from nearly all blossoms sprayed with bacterial suspension and the mean population level was 10^4 CFU/blossom. Population size increased over time and 18 days after application the population reached a level of $1.3x10^6$ CFU/blossom (Figure1). Thus, application of *Eh-24* $^{strR+}$ onto pear blossoms in the orchard resulted in a 100-fold increase in population size 18 days post application.

In vitro tested were used to study the response of *P. agglomerans* strain *Eh 24* to some chemicals, which are routinely sprayed in pear orchards. Mancozeb and copper oxychloride+Maneb decreased the colonial development of strain *Eh24* at doses of 10 and 30 µg/ml, while Copper hydroxide did not adversely affect the colonial development of strain *Eh24*

at dose as high as 100 µg/ml (Table 1). Then, the efficacy of Copper hydroxide on the colonial development of *E. amylovora* was tested in ***in vitro*** conditions. The colonial development of *E. amylovora* decreased by copper hydroxide at the rate of 10 µg/ml and was prevented at a rate of 30 µg/ml.

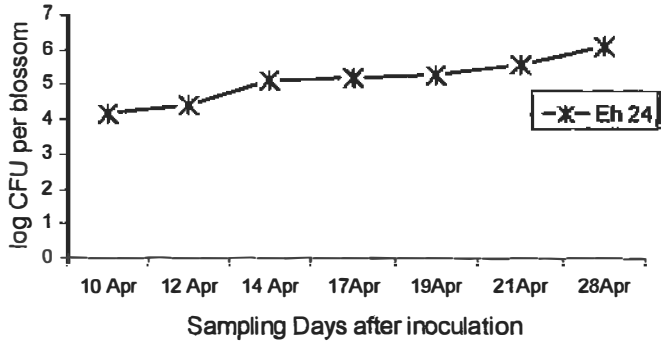

Figure 1. The population sizes of *Pantoea agglomerans* strain *Eh 24*^{StrR} in pear blossoms

Table 1. The effect of some fungicides on the colony development of *Pantoea agglomerans* strain *Eh 24* in ***in vitro*** conditions

Tested chemicals	Colony Counts (CFU/Petri plate) *				
	0 µg/ml	3 µg/ml	10 µg/ml	30 µg/ml	100 µg/ml
Mancozeb	107	110	27	0	0
Copper oxychloride+maneb	107	0	0	0	0
Copper hydroxide	107	102	98	115	106

*There were seven replication plates for each chemical-dose

For biocontrol to be effective, most stigmatic surfaces in an orchard must be colonized by the bacterial antagonists and the population size of antagonists on stigmas must approach the carrying capacity of population size of the tissue (10^5 to 10^6 CFU/blossom) (Wilson et al., 1992; Kearns & Hale, 1993; Johnson & Stockwell, 1998). Johnson et al. (1993) found that early establishment of populations exceeding 10^5 CFU/blossom of bacterial antagonists on pear blossoms suppressed establishment and growth of *E. amylovora*, thereby decreasing disease incidence. In our research, the mean detectable population size of *Eh-24* $^{strR+}$ varied from 10^4 to 10^6 CFU/blossom under field conditions within 18 days following inoculation. The population size of strain *Eh-24* $^{strR+}$ increased in about 100-fold during bloom. These results show that *P. agglomerans* strain *Eh 24* is capable of colonizing pear blossoms. Furthermore, it was found that Copper hydroxide sprays, which were non inhibitory to strain *Eh24*, could be applied to pear trees as a protective measure during winter treatment prior to antagonist application, without

endangering the biocontrol agent. Now, our studies reached the stage of application for patent right of the bio-formulation.

Acknowledgements

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Control of fire blight (*Erwinia amylovora*) with the plant activator BION®

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Abstract: Application of BION® to apple rootstocks M26 reduced the severity of fire blight and the multiplication of causal agent, *Erwinia amylovora*, in the plant tissue. The highest effect was achieved when BION was applied 48 hrs before inoculation. The treatment with BION caused a reduction of symptoms of up to 70%. Reduction in disease severity was correlated with a reduced multiplication of bacteria *in planta*. This effect was demonstrated by PCR.

Key words: Induced resistance, BTH, *Erwinia amylovora*

Introduction

The benzo-(1,2,3)-thiadiazole-7-carbothioic-S-methyl ester (BION®) which was used in our studies, has been tested against several pathogens (Kessman et al., 1994a; Ruess et al., 1995; Oostendrop et al., 1996). A resistance induction of BION was reported against fungi, such as *Erysiphe graminis*, *Septoria* spp., *Pyricularia oryzae*, *Peronospora tabacina*, *Phytophthora* spp., and *Didymella bryonia* (Ruess et al., 1995; Kessmann et al., 1996; Fritz, 1996; Görlach et al., 1996; Ishii et al., 1999), as well as against bacteria, for instance *Erwinia amylovora* (Zeller & Zeller, 1998), *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* (Louws et al., 2001). Efects against viruses were also reported, e.g. cucumber mosaic virus Y (Anfoka, 2000).

Material and methods

M26 apple rootstocks were grown in pots under greenhouse conditions (25±5 °C and 5000- 14000 lux) until shoot length of 20-30 cm. BION (BHT) was used as inducing agent in a concentration of 0.02% by spraying leaves of shoots 48, 72, 96 and 120 hrs before inoculation. Control plants were treated with water. The youngest two leaves of the shoots were cut on the tip and inoculated by dipping into a suspension of $1x10^8$ CFU/ml of a virulent strain of *E. amylovora* Ea7/74 (Zeller & Meyer, 1975).

Determination of bacterial development

For the determination of bacterial multiplication, samples of shoot tips (ca. 1 g) were homogenised with a 0.06% NaCl solution. From each homogenate, a dilution plating (1:10 to 1:1000) was made on modified Miller-Schroth medium (Miller & Schroth, 1972) and incubated for 2 days at 27°C. In addition, it was tested whether the PCR method allowed detecting any changes in the bacterial population after inoculation or treatment with BTH. The homogenate was separated from plant residues by filtration through a sieve followed by centrifugation at 15000 X

g for 10 min. 250 µl solution consisting of 1% (v/v) polyvinyl-propyrolidone (PVPP), 5% (v/v) glycerol and 0.1 M ditreithol (DTT), was added to 1000 µl of the resulting bacterial suspension. The solution was then gently shaken to inhibit probable complications, due to phenolic substances in the PCR reaction (Llop et al., 2000). The bacterial suspension was centrifuged at 13000 X g for 10 min. and the bacterial pellet was suspended in 1 ml distilled water and again centrifuged at 13000 X g for 10 min. The resultant pellet was suspended in 1 ml distilled water, serially diluted with 1% Tween 20 and incubated at 60°C for 10 min. Aliquots of 10 µl were taken for PCR. For the PCR protocol the composition of the reaction mixture and thermal cycle conditions, are listed below.

A. Composition of reaction mixture

10X Qiagen PCR buffer	5 µl
dNTP	2 µl
Primer A	1 µl (pEA 29 A)
Primer B	1 µl (pEA 29B)
Taq DNA polymerase	1 µl
Distilled water	31.5 µl
Sample	10 µl
Total volume	50 µl

B. Conditions of the thermal cycler

Initial denaturation	3 min	94 °C
3-step cycling		
Denaturation:	1 min	94 °C
Annealing:	1 min	52 °C
Extension:	1 min	72 °C
Number of cycles:	28	
Final extension:	10 min	72 °C

For detection of *E. amylovora* by PCR, the method of BERESWILL et al. (1992) was used. As a marker for gel electrophoresis, 0.9 Kb AluI pBR 322 (MBI Fermentas Co. # SM0123) was used.

Results and discussion

The disease index of fire blight in shoots of M26 apple rootstocks was suppressed by BION over a period of 2 weeks from the day of inoculation. The highest effect was achieved by a 48 hrs pre-inoculation treatment. Growth of *E. amylovora* was reduced in BION treated shoots between the 2nd and 11th day after inoculation. The highest decrease of the bacterial population in BION treated plant tissue was observed 7 days after inoculation, with a reduction of 51.2 %.

Evaluation of bacterial populations in planta by PCR

In a greenhouse experiment, 1 day after inoculation no PCR signal was received from all variants. A difference between control and BION treated rootstocks was observed from the 4th until the 7th day after inoculation (Figures 2 and 3).

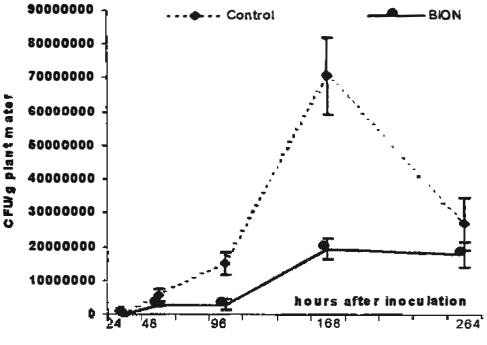

Figure 1. Growth of *Erwinia amylovora* in shoots in BION treated and untreated plants after inoculation

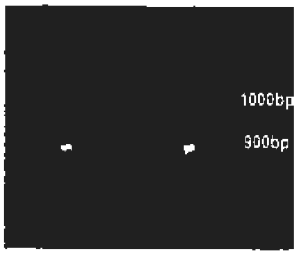

Figure 2. PCR from dilution series of bacterial cultures (Ea7/74 from 10^6 to 10^1) 1. 10^5 2. 10^4 3. 10^3 4. 10^2 5. 10^1 6. 10^6 M=pBR 322 DNA/ AluI Marker, 20

Figure 3. The signal differences on PCR in plant during the experimental period. (PVPP and DTT)

Table 1. Signal differences on PCR in plant during the experimental period

Treatment	Time after inoculation (days)				
	1	2	4	7	11
Control	0	(+)	++	+++	++++
BTH	0	0	(+)	+	+++

0 = No signal, + = very low, ++ = low, ++ = medium, +++ = high, ++++ = very high

In the PCR studies a positive signal (Table 1) was obtained with bacterial concentrations in plant homogenate between 10^6 and 10^3 CFU/ml. The reason for obtaining no signal below a concentration of 10^3 bacteria/ml could be due to inhibitory plant substances (Llop et al., 2000). The pEA29 PCR plasmid has been used successfully in other studies to determine populations of *E. amylovora in planta*. Although we could follow the bacterial multiplication *in planta* by this method, dilution plating was shown to be more sensitive for the detection of low bacterial concentrations.

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Biocontrol of *Erwinia* with bacteriophages

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Abstract: Fifty one bacteriophage isolates of *Erwinia amylovora*, the causal agent of fire blight, were collected from sites in southern Ontario, Canada. Phages were isolated only from sites where fire blight was present. Forty four of these phages were isolated from the soil surrounding infected trees, with the remainder isolated from aerial tissue samples. A mixture of six *E. amylovora* host strains was used to enrich field samples in order to avoid the selection bias of a single-host system. Molecular characterization of the phages with a combination of PCR and restriction endonuclease digestions showed that six distinct phage types were isolated. Ten phage isolates related to the previously characterized *E. amylovora* phage PEal were isolated. The host ranges of the phages revealed that certain types were unable to lyse some *E. amylovora* strains, and that other types were able to lyse the epiphytic bacterium *Pantoea agglomerans*. Biocontrol of *E. amylovora* by the bacteriophages was assessed in a bioassay using discs of immature pear fruit. Twenty-three phage isolates were able to significantly suppress the incidence of bacterial exudate on the pear disc surface. Quantification of the bacterial population remaining on the disc surface indicated that population reductions of up to 97% were obtainable by phage treatment. Use of bacteriophages to control *Erwinia* soft-rot is also discussed.

Key words: bacteriophage, *Erwinia amylovora*, *Erwinia carotovora*, phage morphology

Introduction

Fire blight, caused by the bacterial pathogen *Erwinia amylovora*, is a serious disease of apples and pears. The bacterium infects blossoms and in the presence of susceptible cultivars the pathogen may colonise the developing shoot and older woody tissue. Chemical control strategies against fire blight have been in place since the 1960's and rely primarily on the use of streptomycin during the bloom period. Resistance to streptomycin has been observed in some regions and the need exists for alternate environmentally friendly control strategies.

Biocontrol using bacteriophages relies on the ability of the bacteriophage to infect the host bacterium and use the bacterial machinery to manufacture new virus particles. During the lytic cycle new phage particles are produced and released from the bacterial host cell. The host bacteria are destroyed in the process. The use of bacteriophages as potential biocontrol agents against *Erwinia* soft-rot diseases is being investigated. Greenhouse operations using closed irrigation systems may develop major infestations with soft rot *Erwinia* spp. Soft rot *Erwinia* bacteriophages have been isolated and the process of characterization has been initiated. Biocontrol with bacteriophages in this system may prove successful due to the nature of this pathogen-host system.

Materials and methods

E. amylovora bacteriophages were isolated from soil and cuttings obtained from infected pear and apples using procedures described by Gill (2000) and Adams (1959). *E. amylovora* phages were initially characterised by host range and plaque morphologies. Molecular characterisation was carried out by PCR using primers specific for bacteriophage PEa1 (Schnabel and Jones, 2001). Restriction fragment length (RFLP) analysis using four restriction enzymes was carried out to determine the relatedness of the 44 phage isolates. Biocontrol activity of the *E. amylovora* phages was evaluated using the immature pear fruit assay. Plugs of green fruit tissue were inoculated with the pathogen and the phage isolate, incubated at 26°C and evaluated for symptom development. The bacterial population on the surface of the plug was enumerated following three days of incubation.

Results and discussion

E. amylovora *bacteriophages*

The 51 phage isolates were collected, characterized and screened for biocontrol activity against *E. amylovora*. Molecular characterization indicated that the phages belonged to 6 distinct RFLP groups. The presence of a tail on a phage remains the single most distinguishing feature that provides instantaneous categorization (Ackermann, 1996). Electron microscopy of the phages (Figures 1A-B) revealed that the RFLP Group 1 phages were tailed with contractile tails (Figure 1A), Group 2 were tailed non-contractile (Figure 1B) and Groups 3-6 had very short tails (Figure not shown). The tailed phages of *E. amylovora* belong to the order *Caudovirales* and contain the families *Myoviridae* (contractile tail), *Siphoviridae* (long non-contractile tail) and *Podoviridae* (short tail). Phage isolates that performed well in the plug bioassay are shown in Figure 3. Phages that performed well as biocontrol agents were not restricted to any particular RFLP group. Certain phage isolates were able to reduce the bacterial population to 3% of that found on the plug treated with bacteria alone. Based on the promising bioassay results, phages will be evaluated in a field trial. The results of these trials will determine the feasibility of phage-based biocontrol of the fire blight pathogen.

Soft rot Erwinia *Bacteriophages*

The initial isolation of soft rot-Erwinia phages has been successful. The plaques produced by these phages are pinpoint to 1 mm in diameter. To date, 10 wild type phages have been isolated and work is continuing on the characterization of the phage by host range, electron microscopy and RFLP. Electron micrographs of some isolated phages of *E. carotovora* subsp. *carotovora* reveal that the phages belong to the order *Caudovirales*, families *Siphoviridae* (long non-contractile tail) (Figure 2A,C) and *Myoviridae* (contractile tail) (Figure 2B). Further characterization and phage efficacy trials will be conducted in greenhouses with closed irrigation systems.

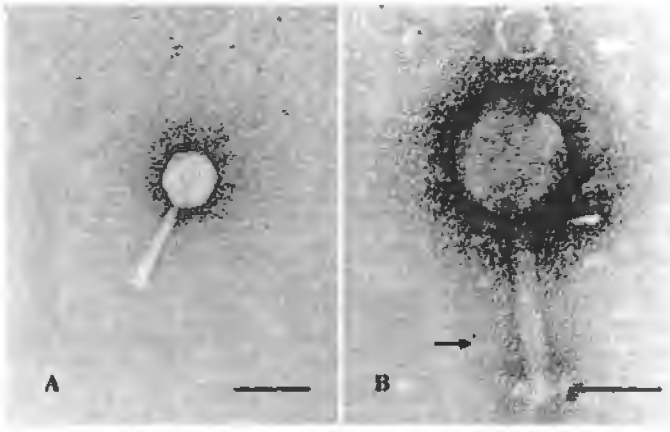

Figure 1. Bacteriophages of *E. amylovora* may belong to the family *Myoviridae* (A). The micrograph shows the phage prior to contraction. The family *Siphoviridae* (B) is characterised by long non-contractile tails. Often decorations are evident at the end of the tail (arrow). Micron marker represents 100 nm.

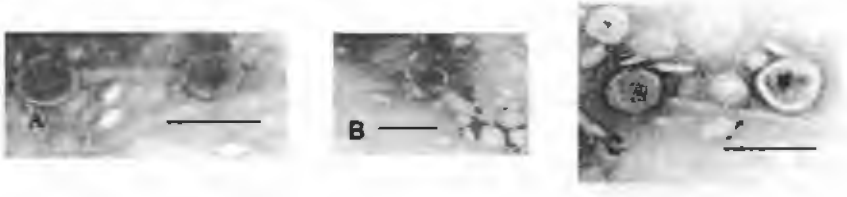

Figure 2. Bacteriophages of *E. carotovora* subsp. *carotovora* belong to the *Siphoviridae* (A, C) and *Myoviridae* (B). Micron marker represents 100 nm.

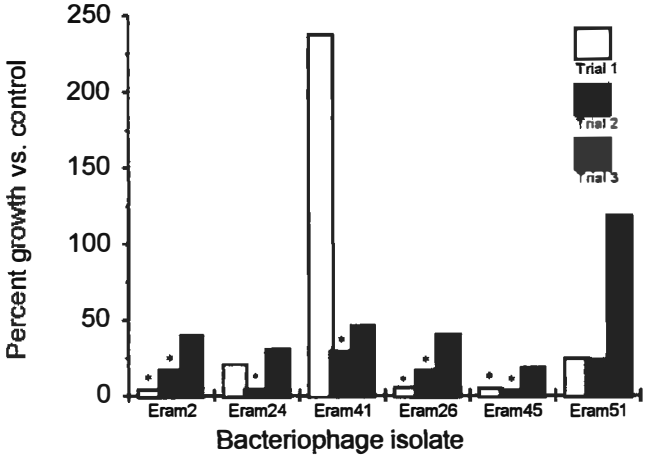

Figure 3. Percentage of bacteria surviving treatment with bacteriophages on plugs of immature pear fruit. Asterisks (*) indicate statistically significant control, $P \leq 0.05$

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Biocontrol of *Erwinia amylovora* with a natural product

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Abstract: A natural product (AkseBio2), obtained from some naturally growing plants in Turkey was assayed as a potential biocontrol agent of *Erwinia amylovora*. Potential activity of the natural product against *E. amylovora* was evaluated by using *in vitro* agar diffusion test, immature pear fruit assay, shoot inoculation, cotoneaster blossom infection and spraying experiments on pear trees. AkseBio2 was more effective than streptomycin and copper oxychloride plus maneb in *in vitro* diffusion test. Ooze formation on immature pear fruits and lesion formation on artificially inoculated shoots were completely (100%) prevented by AkseBio2 and streptomycin (100 µg/ml), whereas copper oxychloride plus maneb suppressed ooze and lesion formation by 80% and 62%, respectively. In an artificial cotoneaster blossom infection test conducted in Germany, blossom infection was decreased by 85% when AkseBio2 was applied and by 50% when plantomycin (0.06%) was applied. AkseBio2 and copper oxychloride plus maneb prevented the appearence of natural disease symptoms of *E. amylovora* at a rate of 64% and 46% on the variety of Santa Maria in Burdur,Turkey, respectively and at a rate of 100% and 69% on the variety of Williams in Isparta,Turkey, respectively.

Key words: AkseBio2, natural product, *Erwinia amylovora*, fire blight

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora*, is a serious disease of pome fruit and ornamentals in the Rosaceae family and wide-spread in the world (Van der Zwet & Keil, 1979). There is no single control measure for fire blight that will totally control the disease. Chemical control of fire blight is diffucult, because copper compounds cause fruit russet, and *E. amylovora* has developed resistance to antibiotics in many important production areas. Alternative control measures are urgently needed for disease suppression. Some elicitors for systemic acquired resistance (SAR) (Lawton et al., 1996), microbial antagonists and plant extracts, are few alternative options for the control of the disease. The main purpose of this work is to test the efficiency of a novel natural compound, AkseBio2, against *E. amylovora*.

Materials and methods

Plant species

A natural product (AkseBio2) was obtained from naturally growing wild plants. The plants were collected from the west Mediterrenean region of Turkey.

Test microorganisms and culture conditions

The bacterial pathogen, *E. Amylovora,* was grown on nutrient agar (NA) (Bectron Dickinson, Cockeysville, MD). Nutrient broth cultures (NB) were grown 24 h at 28 ^{0}C. The bacterial culture was stored in 30% glycerol at –70 ^{0}C.

Agar diffusion test

Antibacterial activity of the natural product was determined by agar diffusion method on NA medium (Johnstone & Thorpe, 1982). The sterile medium in petri dishes was uniformily smeared with culture of *E. amylovora*. Wells (5 mm diameter) were made in the centre of each petri-dish and 30 µl of the natural product was added to each well. For comparison, 30 µl streptomycin and copper plus maneb, were added as well. For each treatment, 5 replicates were maintained. Plates were incubated at 27°C for 48 h and the size of the resulting zone of inhibition was determined.

Immature pear fruit test

The pear fruit test (Billing et al., 1960) was applied for determination the efficacy of the natural product. The fruits were surface sterilized and cut into slices (1 cm thick, 1 cm diameter). The slices were dipped in the natural product suspension and then they were kept for 2 h at 27°C in petri dishes with high relative humidity. Then, a drop of 20 µl containing 10^8 cfu/ml of *E. amylovora*, was placed on each slice and the slices were incubated for 72 h at 27°C. The number of exudate drops on the slice surface was rated from 0 (no symptoms) to 5 (completely covered by exudate). For comparison, slices were dipped in streptomycin solution (100 µg/ml) for 2 h before inoculating with *E. amylovora*.

Shoot inoculation test

Young shoots of Santa Maria pear variety were chosen because of their high susceptbility to *E. amylovora* in shoot inoculation tests. *E. amylovora* (0.1 ml of a 10^8 cfu/ml suspension) and streptomycin (100 µg/ml) were injected to the shoots, 2 cm below the shoot tip. Disease symptomes were evaluated 7 days after inoculation by measuring the lesion diameter, if occurred.

Blossom infection test

Blossoms of cotoneaster were inoculated with *E. amylovora* at the concentration of 7.1×10^7 cfu/ml. Plantomycin (0.06%) was applied for comparison. Evaluation was made after fire blight symptoms were apparent on control blossoms by counting the number of diseased blossom clusters.

Field spraying test

Pear orchards located in the provinces of Burdur (variety Santa Maria) and Isparta (variety Williams) were sprayed with AkseBio2 and copper (0.3%) plus maneb (0.1%). There were 6 trees per treatment. One hundred twigs were tagged on each tree for future evaluation of the disease incidence. Tagged blighted twigs were counted and pruned and the cumulative number of blighted flowers and twigs by the last assesment date was used to determine the efficacy for the AkseBio2 and the Copper and maneb mixture. The statistical software MSTATC was used, and the Duncan's Multiple Range Test was performed for all calculations.

Results

In the agar diffusion test, Aksebio2 and streptomycin had a similar effect on *E. Amylovora*; the inhibition zone of the two compounds were 25 mm and 20 mm, respectively (Table 1). In the immature fruit test, ooze formation was not detected on fruit slices treated with AkseBio2 or with streptomycin, whereas untreated slices (treated with sterile deionized water) were covered with exudates (Table 1). Similar results were obtained in the shoot inoculation test. whereas the avarage lesion lenght in untreated control shoots was 11 cm, lesion were not formed at all in AkseBio2 and streptomycin treated shoots (Table 1). In the blossom inoculation test, disease incidence was reduced by 85% by AkseBio2 and by 50% when Plantomycin was usee (Table 2), without showing any phytotoxicity.

Table 1. Effects of AkseBio2 in agar-diffusion, immature fruit and shoot inoculation test against *Erwinia amylovora*

Treatment	Diameter of the inhibition zone (mm)	Ooze formation*	Lesion lenght (cm)
Control	0 c	+++++	11 a
AkseBio2	25 a	-	0 c
Copper + maneb	14 b	+	4 b
Streptomycine (100 µg/ml)	20 ab	-	0 c

* +: Ooze formation; -: No ooze formation

Table 2. Effects of AkseBio2 in artificial cotoneaster blossom inoculation test against *Erwinia amylovora*

Treatment	Number of blossom clusters		Disease incidence (%)	Disease reduction (%)
	Total	Diseased		
Control	291	76	26	-
AkseBio2	744	27	4	85
Plantomycin (0.06%)	427	55	13	50

AkseBio2 was effective against natural infections of *E. amylovora* in the orchard experiments conducted in both Burdur and Isparta, as compared to the untreated control and the copper + maneb mixture treatments. Disease incidence was reduced by 64% in the Burdur experiment and by 100% in the Isparta experiment (Tables 3 and 4).

Table 3. Effects of the natural product against *Erwinia amylovora* in an orchard experiment conducted in Burdur

Treatment	Number of blighted shoots		Disease incidence (%)	Disease reduction (%)
	Total	Diseased		
Control	600	564	95	-
AkseBio2	600	216	36	64
Copper + maneb	600	324	54	46

Table 4. Effects of the natural product against *Erwinia amylovora* in an orchard experiment conducted in Isparta

Treatment	Number of blighted shoots		Disease incidence (%)	Disease reduction (%)
	Total	Diseased		
Control	600	582	97	-
AkseBio2	600	0	0	100
Copper+maneb	600	186	31	69

Discussion

Results of this study confirm that the natural product, AkseBio2, is effective against *E. amylovora*. Its effectiveness is comparable to that of streptomycin and copper plus maneb. No phytotoxicity for AkseBio2 was detected in all tests including shoot inoculation, blossom infection and field spraying tests under our test conditions.

Growth of the pear trees treated with AkseBio2 was increased as compare to growth of the trees treated with the other products applied in our orchard experiments (results not shown). This may be explained by the increased population on the leaf surfaces of the plants of some epiphytic bacteria including *Pseudomonas flourescens*. *P. flourescens* is an antagonist for *E. amylovora*. It is possible that *P. flourescens* used the natural product as a nutrient for its growth (Baysal & Yegen, 1997).

The natural product may be used as a natural bactericide against *E. amylovora* alone or in combination with an antagonist microorganism or microorganisms. Its use may solve the problems of phytotoxicity resulted from copper sprays and development of *E. amylovora* populations resistant to the antibiotics used for its control. All these may make the natural product, AkseBio2, an atractive alternative measure for the management of *E. amylovora* to be included in integrated pest management programs in Rosaceous plants.

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Biocontrol of *Erwinia amylovora* with antagonistic bacteria

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Abstract: Selected epiphytic bacteria, naturally occurring on rosaceae plants in Turkey, and some other known antagonistic bacteria were assayed for potential biocontrol agents of *Erwinia amylovora*, the causal agent of fire blight. *Bacillus subtilis* AB27 and *Bacillus* BB2 isolates were more effective than others in antibiosis test on ENA (Emmerson Nutrient Agar) medium, although *Erwinia herbicola* was the most effective one in immature fruit assay. In a shoot inoculation test, *Pseudomonas* spp. 39 decreased the average lesion size by 97% by and *E. herbicola* decreased lesion size by 72%. In an cotoneaster blossom infection test conducted in Germany, the epiphytic antagonistic bacteria A22 and *B. subtilis* BD170 reduced blossom infection of *E. amylovora* by 89% and 82%, respectively. Efficacy of selected anatagonists was tested under natural infections in Burdur (variety Santa Maria) and Isparta (variety Williams), Turkey. In the first experiment, the antagonists A14, A22, *B. subtilis* AB27, and *B. subtilis* BD-170 decreased the occurance of natural infection of *E. amylovora* by 73, 82, 82 and 64%, respectively. İn the second experiment, the antagonists A14, *B. subtilis* AB27, *Bacillus* BB2, A22 and *B. subtilis* BD-170 decreased the occurance of natural infection of *E. amylovora* by 87, 87, 75, 81 and 62%, respectively.

Key words: Biocontrol, antagonistic, *Erwinia amylovora*, fire blight

Introduction

Fire blight, caused by *Erwinia amylovora*, is a serious disease of pome fruits (Van der Zwett & Keil, 1979). Control management of fire blight focus on suppression of epiphytic population of *E. amylovora* on flowers by applying antibiotics (streptomycin or oxytetracycline) during bloom. Development of streptomycin resistance populations of *E. amylovora* in many important production are of the world have prompted increased interest in development of alternative control methods. Biocontrol of fire blight may be effective if the antagonists establish high populations prior to the introduction of *E. amylovora* to blossoms (Nuclo et al., 1998; Wilson & Lindow, 1993). Under field conditions, *Pseudomonas fluorescens* A506 and *Erwinia herbicola* C9-1 suppressed establishment and growth of *E. amylovora* on pear blossoms, thereby decreasing disease incidence (Johnson et al., 1993). The purpose of this study was to evaluate some antagonistic bacteria against *E. amylovora in vitro* and *in vivo*.

Materials and methods

Test microorganisms and antagonism

Bacterial cultures were grown on nutrient agar (NA) (Bectron Dickinson, Cockeysville, MD) and Nutrient broth (NB). Cultures were grown for 24 h at 28°C. The bacterial cultures were

stored in 30% glycerol at –70°C. The effects of the antagonistic bacteria were tested on Emerson–Nutrient agar medium (ENA) (Reinhardt & Powell, 1960). *E. amylovora* was included in the medium and the antagonistic bacteria were stroked crosswise on the top of the medium. After 48 hrs. of incubation at 28°C, the growth inhibition zone of the pathogen was determined. The immature pear fruit test (Billing et al., 1960) was applied to test the efficacy of the antagonists as well. Immature pear fruits (Santa Maria) were surface sterilized and cut into slices (1 cm thick, 1 cm diameter). The slices were dipped into an antagonist suspension prepared from 48 hrs-old culture (inoculum density 10^8 cfu/ml). The slices were incubated for 24 hrs. at 27°C in petri dishes with high relative humidity and then they were inoculated by dipping in suspension containing10^8 cfu of *E. amylovora*/ml. Development of ooze drops on the slices was determined after additional 72 hrs. at 27°C, using a scale from 0 (no symptom) to 5 (slice completely covered by exudate). For comparison, fruit slices were dipped in the streptomycin solution (100 μg/ml) for 24 hrs. before being inoculated with *E. amylovora.*

Shoot and blossom tests

Young shoots of Santa Maria pear variety were chosen because of their high susceptbility to *E. amylovora* in shoot inoculation tests. The antagonistic bacteria (0.1 ml of a 10^8 cfu/ml suspension), *E. amylovora (*10^8 cfu/ml) and streptomycin (100 μg/ml) were injected to the shoots, 2 cm below the shoot tip. Disease symptomes were recorded 7 days after inoculation by measuring the lesion diameter, if occurred. The cotoneaster blossoms were treated with the antagonistic bacteria and inoculated with *E. amylovora* at the concentration of $7.1x10^7$ cfu/ml. Plantomycin (0.06%) was applied for comparison. Evaluation was made after fire blight symptoms were apparent on control blossoms by counting the number of diseased blossom clusters.

Field spraying test

Pear orchards located in the provinces of Burdur (variety Santa Maria) and Isparta (variety Williams) were sprayed with the antagonistic bacteria (10^8 cfu/ml) and copper (0.3% plus maneb (0.1%). One hundred twigs were tagged on each tree for future evaluation of the disease incidence. Tagged blighted twigs were counted and pruned and the cumulative number of blighted flowers and twigs by the last assesment date was used to determine the efficacy for the AkseBio2 and the Copper and maneb mixture. The statistical software MSTATC was used, and the Duncan's Multiple Range Test was performed for all calculations.

Results

In the *in vitro* tests, the antagonists *Bacillus subtilis* AB27, *Pseudomonas* spp 39 and A22 had similar effects on *E. amylovora*: they resulted in 10, 8 and 8 mm inhibition zone, respectively. The antagonists *P. fluorescens* and *B. subtilis* BD170 did not have any effect in the *in vitro* tests. *E. herbicola* had a weak effect with 3 mm inhibition zone (Table 1). In the immature fruit test, ooze was not formed on the fruit slices treated with streptomycin, whereas slices not treated with any of the antagonists (control) and those treated with some of the antagonist bacteria were covered with exudates (Table 1). In the blossom infection test, disease incidence was reduced by 89 and 82% by A22 and *B. subtilis* BD170, respectively (Table 2). *B. subtilis* AB27 reduced the disease by 49% (the lowest efficacy) and Plantomycin reduced the disease by 72% (Table 2). The antagonistic bacteria A22 and *B. subtilis* AB-27 reduced the natural infection of *E. amylovora* on Santa Maria by 82 and 82% and on Williams by 81 and 87%, respectively. Disease reduction achieved by copper plus maneb was 46% on Santa Maria and 69% on Williams (Tables 3 & 4).

Table 1. Effects of antagonistic bacteria in agar-diffusion, immature fruit and shoot inoculation test against *Erwinia amylovora*

Treatment	Lesion lenght (cm)	Ooze formations	Inhibition zone (mm)
Control	0 d	+++++	10 a
Erwinia herbicola	2.2 c	+	1 cd
Bacillus subtilis AB27	10 a	++	1 cd
Bacillus subtilis BD 170	0 d	++	3 b
Bacillus subtilis BB-2	10 a	+	2 bc
A14	0 d	+	2 bc
A22	9 b	+	2 bc
Pseudomonas fluorescens A1	0 d	+++	3 b
Plantomycin (0.06%)	NT	-	0 d

$P \leq 0,05$ +: Ooze formation; -: No ooze formation; NT: Not tested.

Table 2. Effects of the antagonistic bacteria in artifi cial cotoneaster blossom inoculation test against *Erwinia amylovora*

Treatment	Number blossom clusters		Disease incidence(%)	Disease reduction (%)
	Total	Diseased		
Control	890	861	97	-
Erwinia herbicola	2085	714	34	65
Bacillus subtilis AB27	1629	806	49	49
Bacillus subtilis BD 170	757	130	17	82
A22	927	102	11	89
Pseudomonas fluorescens P99	1636	941	58	41
Plantomycin (0.06%)	1466	402	27	72

Table 3. Effects of the antagonistic bacteria against *Erwinia amylovora* in an orchard experiment conducted in Burdur

Treatment	Number of blighted shoots		Disease incidence(%)	Disease reduction (%)
	Total	Diseased		
Control	600	576	96	-
Erwinia herbicola	600	324	54	46
Pseudomonas spp. 39	600	270	45	55
Bacillus subtilis BD 170	600	216	36	64
Bacillus subtilis BB2	600	162	27	73
A14	600	162	27	73
A22	600	108	18	82
Bacillus subtilis AB-27	600	108	18	82
Copper+maneb	600	324	54	46

Table 4. Effects of the antagonistic bacteria against *Erwinia amylovora* in an orchard experiment conducted in Isparta

Treatment	Number of blighted shoots		Disease incidence(%)	Disease reduction (%)
	Total	Diseased		
Control	600	558	93	-
Erwinia herbicola	600	300	50	50
Pseudomonas spp. 39	600	150	25	75
Bacillus subtilis BD 170	600	228	38	62
Bacillus subtilis BB2	600	150	25	75
A14	600	78	13	87
A22	600	114	19	81
Bacillus subtilis AB-27	600	78	13	87
Copper+maneb	600	186	31	69

Discussion

Some of the antagonistic bacteria tested in this study showed considerable antagonistic activities against *E. amylovora* under *in vitro* and semi-*in vitro* conditions in pear fruit test. The three antagonistic bacteria, *E. herbicola*, *B. subtilis* AB27, and *Bacillus* BB-2, A14 and A22, were highly effective in shoot inoculation test. *B. subtilis* BD170 and A22 were also effective in the cotoneaster blossom test. The success of these antagonistic bacteria in cotoneaster blossom test was even greater than that of Plantomycin. In the field experiments, A14, A22, *B. subtilis* AB 27 and *Bacillus* BB-2 were highly effective on both Santa Maria and Williams. The effects of the antagonistic bacteria were greater than the effect of the copper plus maneb mixture. With regards to the phytotoxicity problem of copper and copper mixtures and the development of pathogen populations resistant to the antibiotics streptomycin and oxytetracycline, that are commonly used for the control of *E. amylovora*, potential antagonistic bacteria may play an important role in integrated management programme of fire blight disease of Rosaceous plants.

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Development of *Botrytis* species in the presence of grapefruit extract

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Abstract: *Botrytis* species are the most dangerous pathogens in field production of lily, peony and tulip for cut flowers. Amendment of PDA medium with grapefruit extract at 8 µg/ml suppressed the linear growth of *Botrytis* spp. for about 50%. At 40 µg/ml the tested product inhibited germination of spores completely or at least 30%. Additionally, grapefruit extract strongly suppressed the growth of *Botrytis* germ tubes. Spraying the product to plants at concentrations ranging from 165 to 660 µg/ml suppressed the spread of shoot infection of lily and peony. Biological activity of grapefruit extract was higher in the control of *B. paeoniae* than *B. elliptica* and *B. cinerea*.

Key words: *Botrytis*, growth, sporulation, control, lily, peony

Introduction

The use of natural products for the suppression of plant pathogens is considered as an interesting alternative to fungicides due to their lower negative impact on the environment. Among the natural products, grapefruit extract has been found to be active against some bacteria (Woedtke et al., 1999) and pathogenic fungi (Angioni et al., 1998; Caceres et al., 1992; Orlikowski, 2001a,b). Esterio et al., (1992) reported that grapefruit extract at concentration of 1500 µg/ml was as effective in the control of *Botrytis cinerea* on table grape as benomyl or vinclozolin. Amendment of grapefruit extract into media strongly suppressed formation of zoosporangia of *Phytophthora cryptogea* and spore germination (Orlikowski et al., 2001a,b). In this study, development of *Botrytis cinerea*, *B. elliptica* and *B. paeoniae* in the presence of grapefruit extract, as well as biological activity of the product in the control of grey mould, were estimated.

Materials and methods

Biocontrol agent, fungi and plants
Biosept 33 SL, containing 33% of grapefruit extract (GE) from *Citrus* x *paradisi*, supplied by Cintamani, Poland, was used. GE was used at concentrations ranging from 1.6 to 1000 µg/ml. *B. cinerea* from diseased tulip leaf, *B. elliptica* from lily flower and *B. paeoniae* from peony leaf were used. Stock cultures were maintained on PDA at 24°C in the dark. Biological activity of GE in the control of grey mould on leaves and flowers was tested on lily and peony.

Laboratory test
Linear growth of 3 *Botrytis* species in the presence of various GE concentrations was estimated on PDA. The medium amended with 0 (control), 1.6, 8, 40, 200 and 1000 µg of GE/ml, was seeded with 5 mm diam of mycelium disks in 90 mm Petri dishes. Diam of colonies was measured after 3 and 5 - day-incubation. Germination of *Botrytis* spores and growth of germ tubes was observed on water agar amended with GE.

Field trials

Plants with the first disease symptoms were sprayed with GE at concentrations varying from 165 to 660 µg/ml at 7 or 10-day-intervals. Six degree scale was used for estimation of *Botrytis* blight or leaf spot severity. Experimental design was completely randomised with 4 replications and 1 plate or 4 plants in each replication. Trials were repeated at least twice.

Results

Amendment of potato-dextrose agar with GE resulted in the inhibition of *Botrytis* spp. growth (Figure 1). The tested species showed different reaction on GE. *B. cinerea* and *B. elliptica* growth was already inhibited at 1,6 µg of GE/ml and the ED_{50} value was about 40 µg/ml. In case of *B. Paeoniae,* that value was at least twice higher (Figure 1).

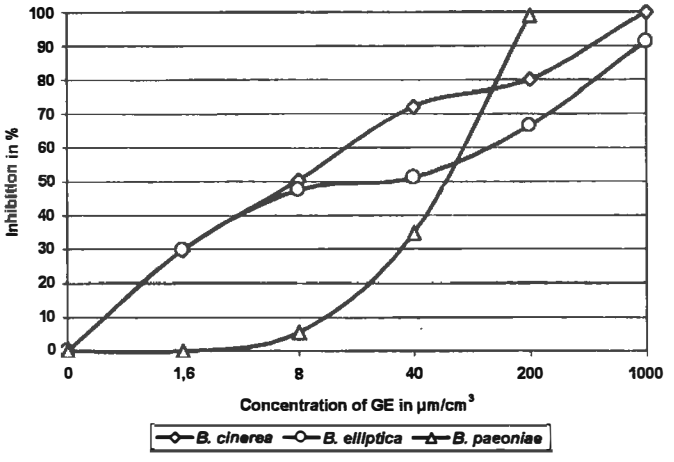

Figure 1. Growth of *Botrytis* spp. on PDA in the presence of grapefruit extract; inhibition of growth in % after 5-day-incubation

Study of spore germination in the presence of GE showed that *B. paeoniae* was more susceptible to the tested product than the other two species (Table 1). The pathogen spores did not germinated already at 40 µg of GE/ml, whereas still about 64% of *B. elliptica* spores germinated at such dose of the product. Analyse of germ tube growth of *Botrytis* spp. confirmed the results obtained for spore germination. Germ tubes of *B. paeoniae* were about 6 times shorter already at 1.6 µg of GE/ml than on nonamended medium (Table 2). Significant decrease of germ tube growth of *B. elliptica* was observed at 40 µg of the product/cml.

In field experiments on peony, weekly application of GE at concentration of 165 µg/ml, decreased disease incidence by 50% as compared to untreated control. Increasing the GE concentration did not improve the control *B. paeoniae* significantly (Figure 2). The control of *B. elliptica* on lily was not satisfactory even when iprodione was used for plant spray (Table 4). GE, however, at dose 165 µg/ml significantly decreased disease development. This effect was comparable to that acheived by iprodione (Figure 2).

Table 1. Spore germination (%) of *Botrytis* spp. in relation to grapefruit extract concentrations after 24 hrs of incubation

Treatment (µg a.i./ml)	*B. cinerea*	*B. elliptica*	*B. paeoniae*
Control	79,7c	88,0d	99,0b
Grapefruit extract (1.6)	83,5c	85,0d	98,0b
Grapefruit extract (8)	78,5c	69,7c	98,0b
Grapefruit extract (40)	13,2b	64,7b	0,0a
Grapefruit extract (200)	0,0a	0,0a	0,0a

Numbers in each column, followed by the similar letters, do not differ significantly ($P \leq 0.05$) as determined according to the Duncan's multiple range test.

Table 2. Germ tubes length (in µm) of *Botrytis* spp. in relation to grapefruit extract concentration

Treatment (µg a.i./ml)	*B. cinerea*	*B. elliptica*	*B. paeoniae*
Control	14,0c	3,5d	21,3c
Grapefruit extract (1.6)	17,7d	3,0d	3,7b
Grapefruit extract (8)	10,9b	2,8c	1,9ab
Grapefruit extract (40)	1,3a	2,1b	0,0a
Grapefruit extract (200)	0,0a	0,0a	0,0a

Numbers in each column, followed by the similar letters, do not differ significantly ($P \leq 0.05$) as determined according to the Duncan's multiple range test.

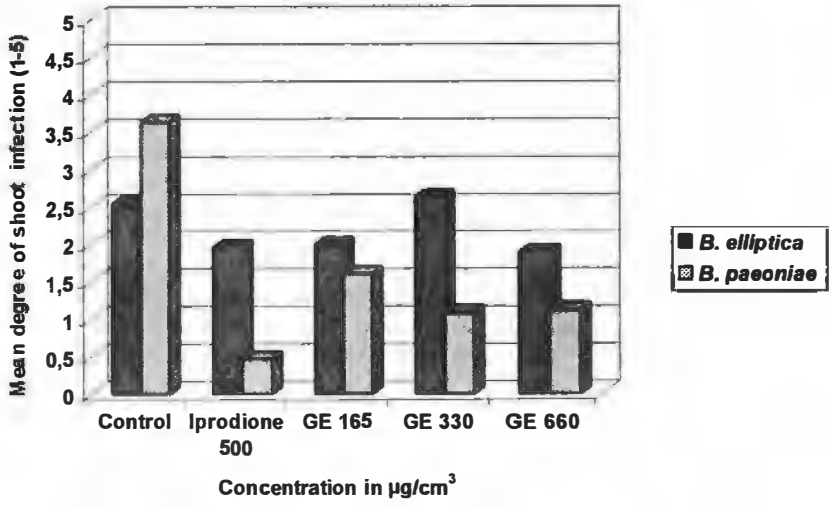

Figure 2. Biological activity of grapefruit extract in the control of *Botrytis* spp. on lily and peony

Discussion

Results obtained indicate a different reaction of *Botrytis* species to grapefruit extract. This confirms results of Caccioni et al. (1995) with reaction of *Penicillium* on citrus fruit essential oils. Strong inhibition of *B. paeoniae* spore germination and suppression of germ tube elongation already at low concentrations resulted in better effect of grey mould control on peonies than on lily. Study of Orlikowski & Skrzypczak (2001) showed satisfactory effect of grapefruit extract in the control of *B. tulipae*. The product was even better than iprodione when applied at dose 660 µg/ml. Caccioni et al. (1995) suggested that among the many components included in citrus fruits, oxygenated monoterpenes have antifungal activity. Besides direct action of grapefruit component on fungi, essential oils of citrus fruits might represent a pre-formed defence barrier *in situ* interfering greatly in host-pathogen relations. Lignin-like materials and phytoalexins (Caccioni et al., 1998) may integrated their activity.

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Biocontrol of *Botrytis cinerea* infection of tomato in unheated polytunnels in the North East of Scotland

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Abstract: A randomised block field trial was constructed to test the ability of several biocontrol agents (*Brevibacillu brevis*, Milsana® and Trichodex®) independently and in combination to control *Botrytis cinerea* in the highly variable but cooler environment of unheated polytunnels in the North East of Scotland. The only biocontrol agent to significantly reduce leaf disease severity at all was *B. brevis*, which reduced disease by around 30% ($p=0.1$). Stem disease was not reduced by any of the treatments used. Fruit yields were significantly improved ($p=0.05$) in plants treated with *B. brevis* alone or in combination with Milsana®. It is thought that the environmental conditions prevalent, which were ideal for *B. cinerea,* rendered the biocontrol agents ineffective except for *B. brevis* which operated at a lower efficacy than has been observed previously. In addition, the effect of cultivar on results is discussed.

Key words: biocontrol, *Botrytis cinerea*, grey mould, Trichodex®, *Trichoderma harzianum*, Milsana®, *Brevibacillus brevis*, tomato, field trial

Introduction

This research is part of an EU project (BIOCOMBI, FAIR CT98-4413) in which a number of fungal, bacterial and plant extract biocontrol agents are used alone or in combination against important diseases of greenhouse crops. The object of the research presented here was to determine the most effective of these agents, both separately and in combinations, for biocontrol of *Botrytis cinerea* (grey mould) infection of tomato in unheated polytunnels in Aberdeenshire, Scotland. Treatments used in this study included the plant extract Milsana®, derived from the Giant Knotweed, *Reynoutria sachalinensis*; TRICHODEX®, a commercial formulation of the fungus *Trichoderma harzianum* T39 (Elad et al., 1995) and the bacterium *Brevibacillus brevis* (formerly named *Bacillus brevis*) which has been developed for biocontrol purposes (Edwards et al., 1994).

Materials and methods

A randomised block field trial was designed incorporating 6 plots (1 per treatment) of 12 tomato plants (cv. 'Moneymaker') in each of four blocks. Treatments used were: *Brevibacillus brevis* alone; *B. brevis* and Milsana® (VP2001 1.4%) combined; Milsana® (VP2001 1.4%) alone; TRICHODEX® (4 gl^{-1}) alone; TRICHODEX (4 gl^{-1}) and Milsana® (VP2001 0.35%) combined and a control treatment of sterile distilled water (SDW).

B. brevis was cultured in tryptic soy broth (TSB) in a rotary incubator (150 rpm, 37 °C) for 6 days and used in whole culture form. TRICHODEX ® is a commercial formulation (Makhteshim Agan Ltd, Israel) of *Trichoderma harzianum* T39 and was diluted in SDW. Milsana concentrate was diluted in the appropriate volume of SDW, TRICHODEX ® solution or *B. brevis* culture immediately before spraying and mixed thoroughly. Treatments were applied once per week to run off.

Grey mould severity was scored weekly by estimating percentage diseased leaf area and a disease progress curve was constructed from which the Area Under the Disease Progress Curve (AUDPC) was calculated (Campbell & Madden, 1990). Length of stem lesions for each plant were recorded. Fruit was harvested when necessary and total yields as well as marketable yields (Class 1 fresh market) were recorded. Ambient temperature and humidity were monitored using Campbell CR10X data loggers (Campbell Scientific Ltd) and temperature/humidity probes (Campbell Scientific Ltd). Disease data (final stem lesion length, final leaf disease severity, AUDPC) was subject to analysis of variance (ANOVA) and Fisher's LSD test. It was necessary to normalise final leaf disease severity by square root transformation prior to analysis.

Results and conclusions

Disease progress was similar for all treatments (Figure 1). The only treatment which resulted in significantly lower leaf disease than control plants was *Brevibacillus brevis* (p=0.1) which reduced grey mould disease by around 30%.

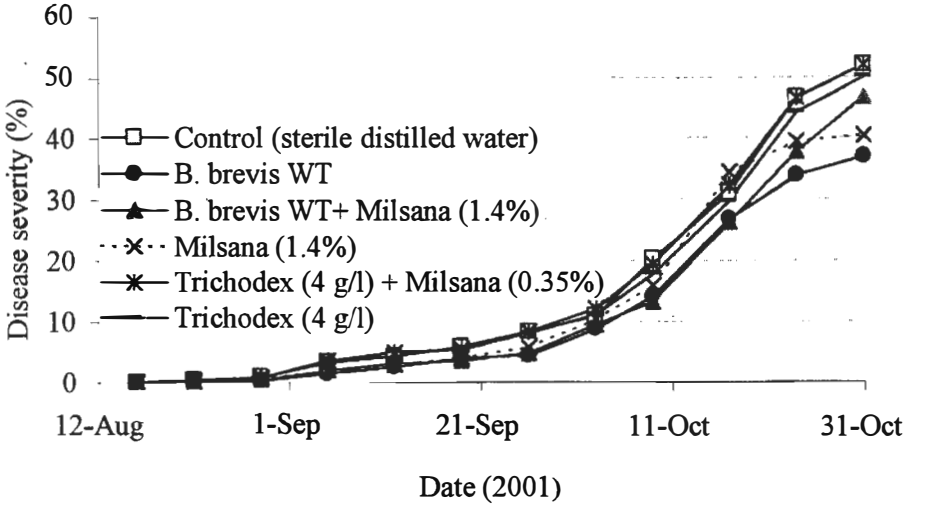

Figure 1. Disease progress in leaves based on estimated percentage diseased leaf tissue.

The efficacy of *B. brevis* in controlling grey mould leaf disease has previously been demonstrated in similar environments. However the treatment here was neither as effective nor was its efficacy as statistically significant, as in previous studies where 44% (P=0.05) disease reduction was achieved (Seddon et al., 2000). Former studies, although in a similar

environment (polytunnels in the North East of Scotland), involved F1 hybrid (cv. 'Shirley') tomato plants and it may be that in such a variable environment, the consistency, superior disease resistance and vigour provided by F1 hybrid plants allows better biocontrol and less variability in results. Previous control plants had half the final disease severity of those in this

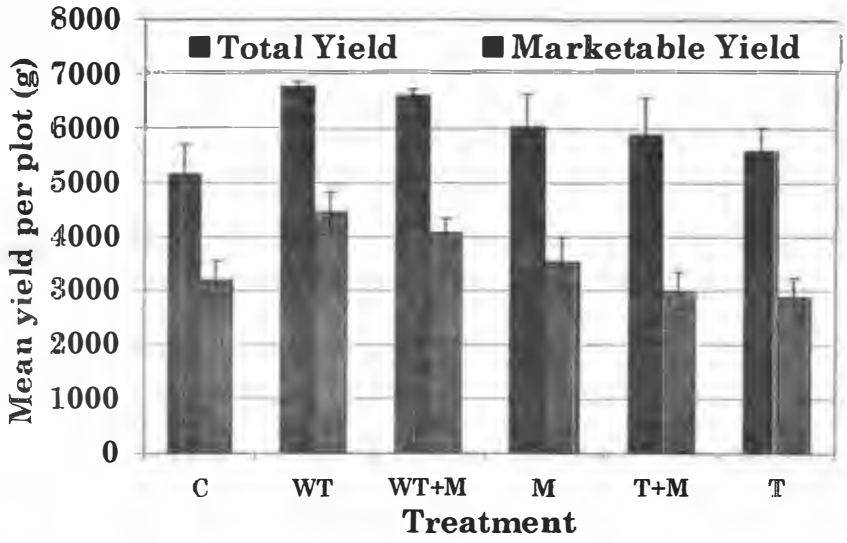

Figure 2. Tomato fruit yields (±SE). C, control (SDW); WT, *Brevibacillus brevis*; WT+M *B. brevis* and Milsana®; M, Milsana®; T+M, Trichodex® and Milsana®; T, Trichodex®

study, possibly because of the cultivar used, and the lower disease pressure may have favoured more effective biocontrol activity. Milsana® or Trichodex® alone did not reduce disease significantly.

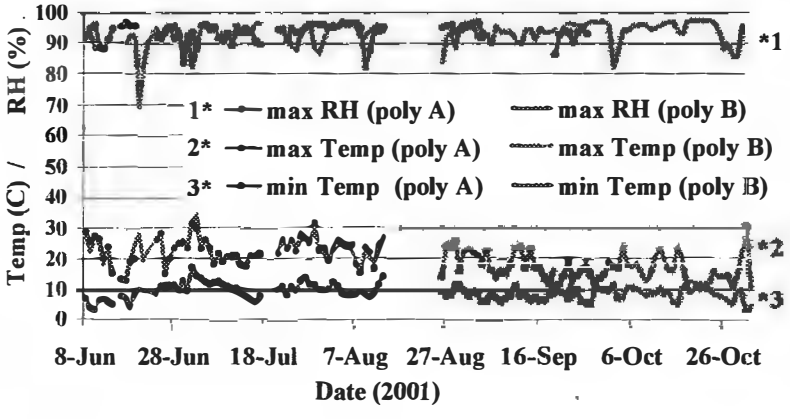

Figure 3. Minimum (min) and maximum (max) daily temperatures (Temp) and maximum relative humidity (RH) in each polytunnel (poly A and poly B)

Use of *B. brevis* alone resulted in higher fruit yields (Figure 2) as did Milsana® and *B. brevis* together which suggests that the increased yields in the combination was due solely to *B. brevis* especially since Milsana® alone had no effect on yields. The standard error (Figure 2) of fruit yields was also remarkably low in plants treated with WT and as such, yields were more consistent between plots. The marketable yield (fresh Class 1) achieved with *B. brevis* was also highest at 66% of the total yield (Figure 2) as compared to 62% of fruit from control plots, 58% of fruit from Milsana plots and 51% of fruit from all treatments containing TRICHODEX ®.

Grey mould disease severity increased most in September and October as shown in Figure 1. This can be compared to Figure 3, showing the associated maximum and minimum daily temperatures and maximum relative humidity in the polytunnels. Temperatures at this time rarely rose above 20°C. Such conditions were suitable for both the pathogen and, less so, *B. brevis* which is reported to have biocontrol activity at 16 – 24°C (Markellou, 1999), but not for *T. harzianum* T39 which is well documented to be most active above 20°C (Hjeljord et al., 2000).

Thus, in these field trials in the environment of unheated polytunnels in the North East of Scotland, TRICHODEX ® appears to have been rendered ineffective by virtue of environmental conditions unfavourable for its germination and activity whereas *B. brevis* remains effective albeit with a reduced efficacy than previously observed (Seddon et al., 2000). This illustrates the importance of either selecting biocontrol agents to suit the anticipated environmental condition or having the ability to manipulate environmental conditions suitable for biocontrol agents requiring certain conditions for activity.

Acknowledgements

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Influence of temperature on antimicrobial activity of grape volatiles on *in vitro* growth of *Botrytis cinerea*

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Abstract: The aim of the research was to study the influence of temperature on inhibition of *Botrytis cinerea* growth by volatile substances produced by grapes of the "Isabella" variety of the *Vitis labrusca* species, in *in vitro* tests. The anti-fungal activity of the volatile substances under different temperatures was determined quantitatively in a series of *in vitro* experiments using a bioassay based on the closed Mariotte system. The following treatments were included in the experiments: (i) volatile substances produced by grapes of the resistant variety "Isabella" (*V. labrusca*); (ii) volatile substances produced by grapes of the susceptible Greek variety "Roditis" (*V. vinifera*); and (iii) no volatile substances at all. The results revealed an inhibitory activity of the volatile substances from the resistant variety of grape on sporulation and creation of sclerotia of *B. cinerea*; volatile substances from the susceptible variety stimulated sporulation of the fungus. The inhibitory and stimulating activities of the volatile substances were more pronounced at the temperature of 21°C than at 10 or 0°C.

Key words: *Botrytis cinerea,* biological control, volatiles, grapes, "Isabella"

Introduction

Botrytis cinerea is an important pathogen of stored and transported fruits and vegetables. In most hosts and under humid conditions, the pathogen sporulates abundantly on the surface of infected areas. Kulakiotu (2001) found that volatile substances emitted by grapes of the "Isabella" variety (*Vitis labrusca* species) inhibited sporulation of the fungus. In was found further that volatile substances emitted by grapes of the "Isabella" variety suppressed sporulation of *B. cinerea*, while volatile substances produced by grapes of the "Roditis" variety (*V. vinifera* species) stimulated it.

One of the most important a-biotic factors for both the growth of *B. cinerea* and the emission volatile substances is temperature. The aim of this research was to study the influence of temperature on the antimicrobial activity of volatile substances produced by grapes of the "Isabella" variety. The long-term goal of this research is to evaluate whether these substances could be used as potential biocontrol agents for post-harvest suppression of the fungus.

Material and methods

The antimicrobial activity of volatile substances on the growth of *B. cinerea* was determined quantitative in *in vitro* experiments. Experiments were performed using a bioassay based on the closed Mariotte system (Sfakiotakis, 1972). Treatments included in the experiment were:

(i) volatile substances produced by grapes of the resistant variety "Isabella" (*V. labrusca*), (ii) volatile substances produced by grapes of the susceptible variety "Roditis" (*V. vinifera*), and (iii) no volatile substances at all. The antimicrobial activity of the volatile substances was tested at temperatures of 0, 10 and 21°C.

Results

The results revealed that volatile substances from the resistant variety of grapes inhibited the sporulation of the fungus (Figure 1) and creation of sclerotia (Figure 2). On the other hand, volatile substances from the susceptible variety stimulated sporulation of the fungus (Figure 1). The inhibitory and stimulating activity of the volatile substances from of the resistant and susceptible variety, respectively, were more pronounced at the temperature of 21°C and at 10 or 0°C (Figures. 1 and 2).

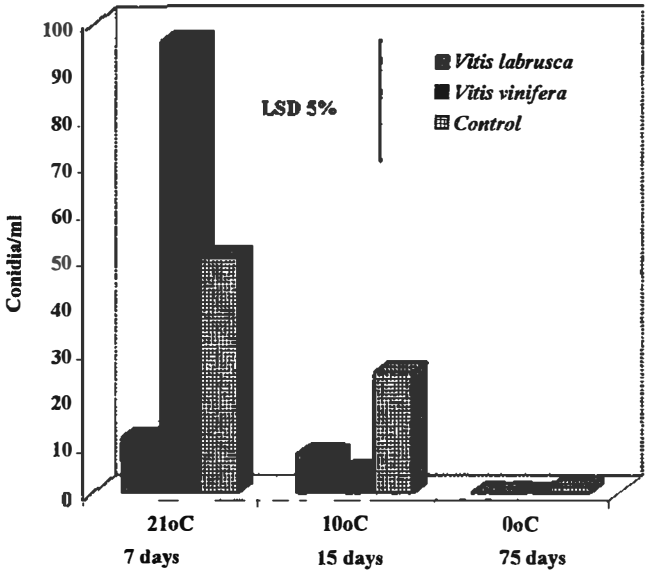

Figure 1. Influence of volatile substances produced by grape berries of the varieties "Isabella" (*V. labrusca*) and "Roditis" (*V. vinifera*), on the production of conidia by *Botrytis cinerea*, at various temperatures. Effects on sporulation were determined at various times after inoculation in a closed Mariotte system. $LSD_{5\%} = 24.0$.

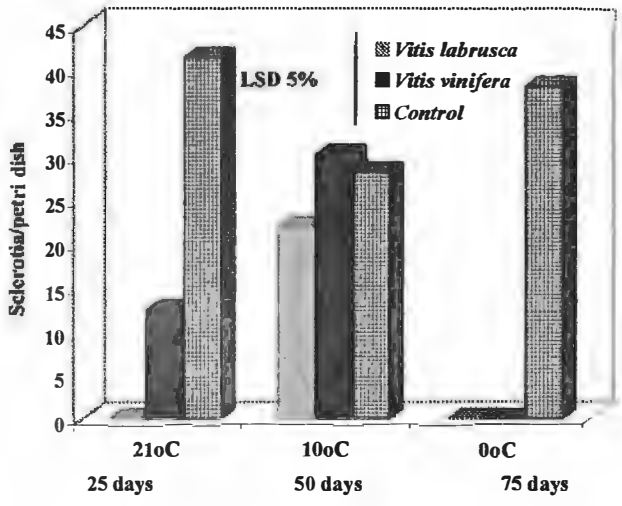

Figure 2. Influence of volatile substances produced by grape berries of the varieties "Isabella" (*V. labrusca*) and "Roditis" (*V. vinifera*) on the formation of sclerotia by *B. cinerea,* at various emperatures. Effects on sclerotia production were determined at various times after inoculation in a closed Mariotte system. $LSD_{5\%} = 9.7$.

Discussion

Temperature of 21°C was the most favorable for expression of the antifungal activity of the volatile substances emitted by the "Isabella" variety. The fact that the volatiles of Isabella inhibited sporulation of the fungus, while those of Roditis stimulated it, may suggest that the expression of the resistance of "Isabella" and the susceptibility of "Roditis" to *B. cinerea* are related to the inhibitory and stimulating activity of the volatile substances of their grape berries. Nevertheless, the possible involvement of other factors should not be excluded. It also intimates the important role of volatile substances as inhibitors or stimulators of an organism (Fries, 1973; French, 1985).

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Influence of temperature in antimicrobial activity of grape volatiles on *in vivo* growth of *Botrytis cinerea*

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Abstract: The aim of the research was to study the influence of temperature on antimicrobial activity of volatile substances produced by grapes of the "Isabella" variety of the *Vitis labrusca* species. The post-harvest control of *Botrytis cinerea* on berries of *V. vinifera* was tested quantitatively using a bioassay based on the closed Mariotte system. The results confirmed the antimicrobial activity of the volatile substances from the grapes of the "Isabella" variety as they limited the incidence of infection and reduced considerably the amount of inoculum. The antimicrobial activity was more effective at temperature of 21°C that at 10 or 0°C.

Key words: *Botrytis cinerea,* post harvest biological control, volatiles, grapes, "Isabella"

Introduction

The most important cause of post-harvest rots of grapes is the fungus *Botrytis cinerea,* which can cause severe losses especially under wet conditions. With the exception of sulfur dioxide treatment of table grapes after harvest, there are no commercially acceptable or safe post-harvest treatments to suppress *B. cinerea* during the storage of grapes (Archbold et al., 1997). Kulakiotu et al. (2002) found that natural compounds of grape berries of the "Isabella" variety of the *Vitis labrusca* species possess *in vitro* antifungal activity against *B. cinerea.* As such compounds may have potential as post harvest fumigants, the research continued at the level of *in vivo* experiments on grapes.

The aim of this research was to study the influence of temperature in the antimicrobial activity of volatile substances produced by grapes of the "Isabella" variety (*V. labrusca* species). Effects were determined on growth of *B. cinerea* on fruits of *V. vinifera.* The long-term goal of this research is to evaluate whether these substances could be used as potential biocontrol agents for post-harvest suppression of the fungus.

Material and methods

The antimicrobial activity of the volatile substances, on the growth of *B. cinerea* was determined quantitative in *in vivo* experiments. Experiments were performed using a bioassay based on the closed Mariotte system (Sfakiotakis, 1972). The antimicrobial activity was tested in temperatures of 0, 10 and 21°C. The volatile substances used were produced by grapes of the "Isabella" variety, and the development of *B. cinerea* was tested on clusters of the Greek variety "Roditis" (*V. vinifera*).

Results

Volatile substances from the grapes variety "Isabella" reduced the incidence of *B. cinerea* infection (Figure 1) and the rate of *B. cinerea* sporulation (Figure 2) on clusters of the variety Roditis". The antimicrobial activity was more pronounced at the temperature of 21°C than at 10 or 0°C.

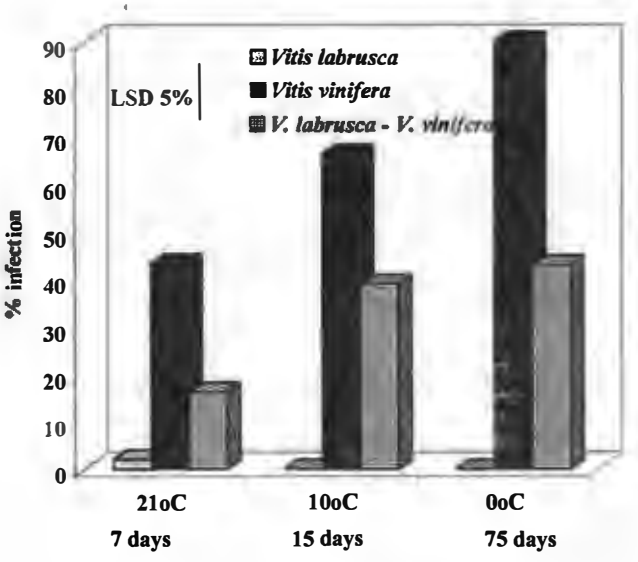

Figure 1. Influence of temperature on *Botrytis cinerea* infection of grape berries of the varieties "Isabella" (*V. labrusca*), "Roditis" (*V. vinifera*) or "Roditis" in the presence of "Isabella". Effects on infection were determined at various times after inoculation in a closed Mariotte system. $LSD_{5\%} = 12.2$.

Discussion

This research presents experimental data which demonstrate the strong antifungal activity of volatiles substances produced by grapes of the "Isabella" variety against *B. cinerea.* These substances reduced the incidence if infection and the rate of sporulation. The antifungal activity of the volatiles produced by "Isabella" were apparent at all temperatures tested (0, 10 and 21°C), but it was most pronounced at 21°C. As the volatiles substances resulted in less infected grape berries and also in less sporulation on each infected berry, the total effect on yield loss were two-fold. This also led to a reduction in the inoculum potential of the pathogen.

The antimicrobial abilities of grape volatiles produced by the "Isabella" variety are of particular interest for post-harvest application. However, more research is needed for identification of the natural volatile compounds that are responsible for suppression of gray mold during the post-harvest storage of grapes. The demand for natural, low toxicity volatiles is increasing. These compounds may be used as components in integrated control strategy,

which, would enable a more acceptable and less environmentally damaging form of agriculture.

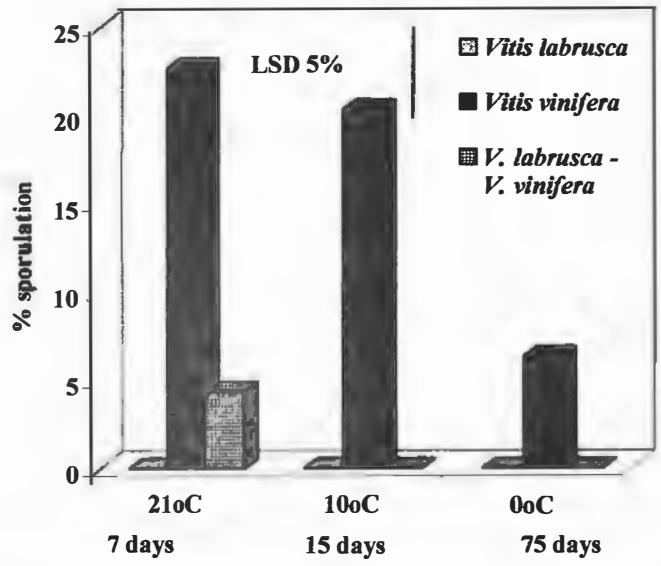

Figure 2. Influence of temperature on *Botrytis cinerea* sporulation on grape berries of the varieties "Isabella" (*V. labrusca*), "Roditis" (*V. vinifera*) or "Roditis" in the presence of "Isabella". 7 Effects on infection were determined at various times after inoculation in a closed Mariotte system. $LSD_{5\%}$: = 4.76.

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Use of *Trichoderma* spp. for biocontrol of *Colletotrichum acutatum* (anthracnose) and *Botrytis cinerea* (grey mould) in strawberry, and study of biocontrol population survival by PCR

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Abstract: *Trichoderma* isolates are known for their ability to control plant pathogens. In a previous study, we showed that various isolates of *Trichoderma*, including isolate T39 from the commercial biocontrol product TRICHODEX, were effective in control of anthracnose (*Colletotrichum acutatum*) and grey mould (*Botrytis cinerea*) in strawberry, under laboratory and greenhouse conditions. Three selected *Trichoderma* strains (T-39, T-161 and T-166) were assessed in a semi-commercial field experiment using different timing application and dosage rates for suppression of strawberry anthracnose. All possible combinations of single, double or triple mixtures of the *Trichoderma* strains, applied at 0.4% and 0.8% concentrations, and at 7 or 10 day intervals, were tested. Anthracnose severity (i.e., the number of diseased plants, apexes and petioles), was reduced by all possible combinations of the applied *Trichoderma* strains compared to untreated controls. A similar field experiment was conducted to evaluate the efficacy of these strains for control of grey mould. Reduction of grey mold was highest when the *Trichoderma* strains were applied at the 2-day intervals at 0.4% concentration. In experiments attempting to determine the survival dynamics of populations of the *Trichoderma* isolates applied in mixtures, PCR using repeat motif primers was conducted. In these experiments, strawberry leaves were treated with a mixture of the isolates and dilution plating was done on plates containing a *Trichoderma*-selective medium. The strains were identified according to PCR band patterns typical for each *Trichoderma* isolate and compared to applications of sole isolates. A molecular approach, using PCR methodology, has been initiated for species identification of selected and reference *Trichoderma* strains.

Keywords: anthracnose, *Botrytis*, *Colletotrichum*, PCR, strawberry, *Trichoderma*

Introduction

Anthracnose and grey mould caused by *Colletotrichum acutatum* Simmonds and *Botrytis cinerea* Pers.:Fr., respectively, are two of the major fungal diseases of strawberry occurring worldwide (Elad et al., 2000; Freeman & Katan, 1997). *Trichoderma* isolates are known for their ability to control plant pathogens. In a previous study, we showed that various isolates of *Trichoderma*, including isolate T39 from the commercial biocontrol product TRICHODEX (O'Neill et al., 1996), were effective in controlling anthracnose and grey mould in strawberry, under laboratory and greenhouse conditions (Freeman et al., 2001). In this work, selected *Trichoderma* strains were further assessed for biocontrol of anthracnose and grey mould in a semi-commercial field experiment using different timing application and dosage rates.

Survival dynamics of populations of the *Trichoderma* isolates applied in a mixture is of importance to determine fitness of certain isolates. Survival may be quantified by the serial dilution method, however, morphologically it is not possible to distinguish between applied

strains in a mixture. A molecular approach using arbitrarily-primed PCR was assessed to quantify the different isolates. In addition, species identification of all the original 72 *Trichoderma* strains used in this study (Freeman et al., 2001) compared to reference strains, has been initiated using different molecular tools (Hermosa et al., 2000).

Materials and methods

Three selected *Trichoderma* strains, including isolate T-39 (TRICHODEX), strain T-161 and strain T-166, were applied in a semi-commercial field experiment using different timing application, dosage rates and mixtures for reduction of strawberry anthracnose and grey mould. All possible combinations of single, double or triple mixtures of the *Trichoderma* strains, applied at 0.4% and 0.8% concentrations, and at 7 or 10-day intervals, were applied. The plants were spray treated by conidia (10^6 /ml) of the *Trichoderma* isolates and infected by spraying conidia of *B. cinerea* or *C. acutatum* (10^5 and10^6/ml, respectively) from 2 weeks old cultures. Disease severity was assessed on a weekly basis.

Survival of *Trichoderma* isolates (T-39, T-105, T-161 and T-166) alone or in combination was assessed on strawberry leaves applied in a drop. The leaves were washed and conidia were serially diluted on plates. The resulting colonies were identified according to PCR band patterns typical for each *Trichoderma* isolate and compared to applications of the individual isolates. Species determination of 77 *Trichoderma* strains including 5 reference strains (received from Prof. E. Monte, Salamanca, Spain) was conducted by arbitrarily-primed PCR using repeat motif primers such as $(GACA)_4$, under the conditions previously described (Freeman & Katan, 1997), and analyzed by the NTSYS software (Rohlf, 2000).

Results and discussion

Anthracnose and grey mould reduction

All combinations of single, double or triple mixtures of *Trichoderma* strains, applied at 0.4% and 0.8% concentrations, and at 7 or 10 day intervals, effectively reduced anthracnose severity (Figure 1). The most pronounced effect was obtained by the 7-day application at 0.8% concentration.

Reduction of grey mould was highest when the *Trichoderma* strains were applied at the 2-day intervals at 0.4% concentration (Figure 2). It was shown that under semi-commercial conditions anthracnose and grey mould in strawberry could be reduced by application of *Trichoderma* isolates and a combination of strains. The time period between applications is important and 2-7 days appeared to be the desired range, whereas 0.8% concentration was preferable.

Survival experiments

Survival of the isolates applied alone declined to undetectable levels after 14 days incubation on leaves (data not shown). Survival of isolates in the mix, calculated according to PCR, also declined to undetectable or low levels after 14 days. However, strain T-105 appeared fitter and survived at higher concentrations than the other isolates.

It can be concluded that it is possible to determine survival and fitness of isolates of *Trichoderma* applied in a mixture using PCR technology and certain isolates appeared to survive better in a mix than others indicating diversity in fitness.

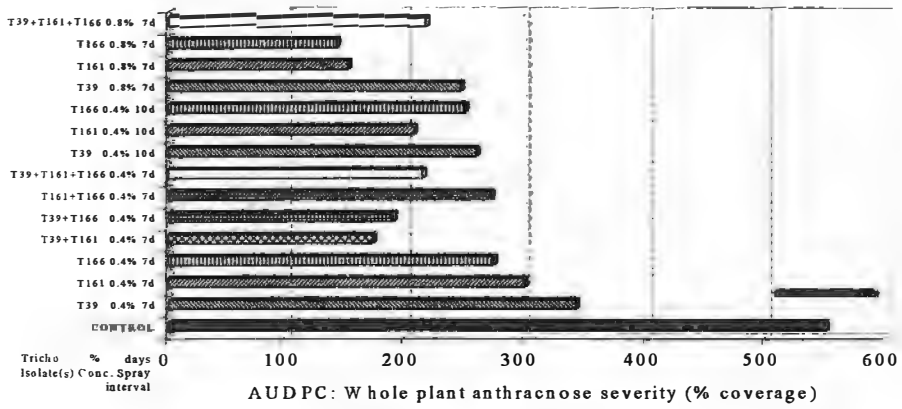

Figure 1. Anthracnose (*Colletotrichum acutatum*) severity expressed as Area Under the Disease Progress Curve (AUDPC). The bar indicates the lowest significant difference (LSD) between treatments ($P \leq 0.05$).

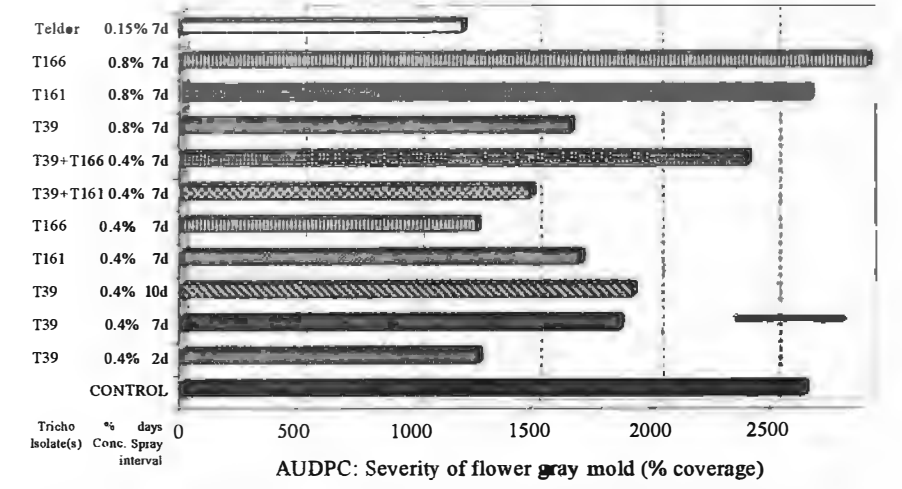

Figure 2. Grey mould (*Botrytis cinerea*) severity expressed as Area Under the Disease Progress Curve (AUDPC). The bar indicates the lowest significant difference (LSD) between treatments ($P \leq 0.05$).

Molecular characterization of Trichoderma *isolates*

Differentiation of the *Trichoderma* isolates to species according to ap-PCR seemed reliable and separated between the reference isolates, also placing isolate T-39 within the *T. harzianum* cluster (Figure 3). AP-PCR grouped the isolates according to species, however, further studies involving ITS sequencing are required to obtain a more reliable phylogenetic tree.

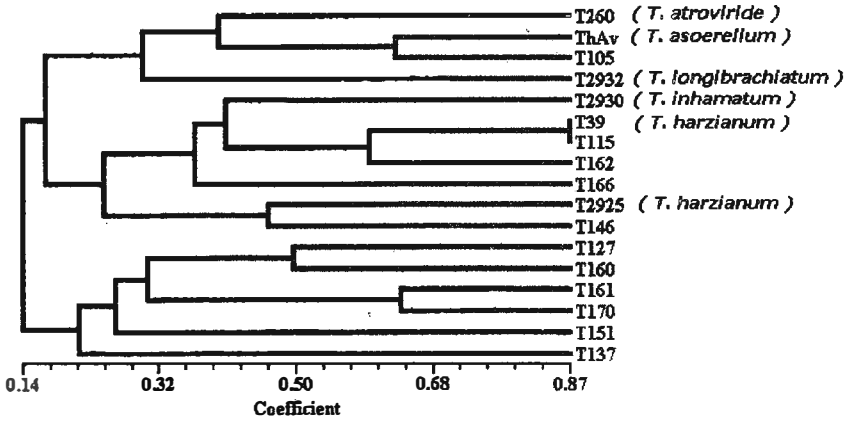

Figure 3. Phylogenetic tree of representative *Trichoderma* isolates using the NTSYS software, UPGMA clustering analyses.

Acknowledgements

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Combinations of biocontrol agents and Milsana® against powdery mildew and grey mould in cucumber in Greece and the Netherlands

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Abstract: Five greenhouse trials were conducted in Greece and the Netherlands to study the improvement of effectiveness of Milsana® against powdery mildew and *Botrytis cinerea* (only in the Netherlands) when combined with other control methods. Cucumber plants were treated with either Milsana®, *Pseudozyma flocculosa* (syn.*Sporothrix flocculosa)* or *Brevibacillus brevis* WT as stand-alone treatments or in combinations. All treatments with Milsana® significantly reduced powdery mildew severity in all trials. Milsana® alone or in combination with *P. floccculosa* increased the number and/or weight of harvested fruits in Greece. In the Netherlands, the three control agents generally reduced powdery mildew and in one trial, *B. brevis*+Milsana® was better than either one alone. *B. cinerea* lesions were not reduced by any of the treatments but in 2000, the number of dead plants was reduced by *B. brevis* combined with Milsana®. Effects of the treatments on yield were variable.

Key words: *Brevibacillus brevis, Pseudozyma floccculosa*, combinations, efficacy, yield

Introduction

Over the years, the pathogens *Sphaerotheca fuliginea* (Schlecht) Pollaci and *Botrytis cinerea* Pers.:Fr. in cucumbers have been controlled mainly by chemical fungicides but recently reports have highlighted the potential of biological and other prophylactic methods as alternative strategies for management of these diseases. Ethanolic or aqueous extracts from the giant knotweed *Reynoutria sachalinensis* (F. Schmidt) Nakai were found to be effective against powdery mildew fungi in cucumbers, by induction of resistance in the plants to the pathogens (Herger & Klingauf, 1990,; Konstantinidou-Doltsinis & Schmitt, 1998). Also, the yeast-like fungus *Pseudozyma flocculosa* was proved to be a powerful antagonist of powdery mildew fungi in roses and cucumbers (Bélanger & Labbé, 1995; Dik et al., 1998). Biocontrol is also an alternative option to reduce *Botrytis* infection and has shown to be effective in many crops (Elad et al., 1996). *Brevibacillus brevis* wild type (WT) was proved to be a potential biocontrol agent against *B. cinerea, S. fuliginea* and *Pythium* spp (Seddon et al., 2000). However, commercialization of biocontrol methods is thus far limited to a few products. It is well known that biocontrol agents often become less effective under certain biotic or abiotic conditions hence it is necessary to integrate biocontrol with other measures. The aim of an EU-funded project (FAIR CT-98-4413; Biocombi) was to enhance plant health by induction

of resistance (with *R. sachalinensis*) and by integration of well defined and tested antagonistic microorganisms (*P. flocculosa* and *B. brevis* WT) that inhibit conidial germination and mycelial development.

Materials and methods

Cucumber plants of the cv. Sandra (Greece, GR) and cv. Jessica (The Netherlands, NL) were grown according to local normal practice. The plots were arranged according to complete randomised block design with 4 replicates (each plot consisted of 5 –16 plants). In five greenhouse trials (two in GR, three in NL), the following treatments were applied: 1) water; 2) Milsana® (0.3-1.5%); 3) *Brevibacillus brevis* wild type WT (10^6-10^7 spores/ml); 4) *Pseudozyma flocculosa* (5 ml l^{-1}); 5) *B. brevis* WT + *P. flocculosa* 6. Milsana® + *B. brevis* WT; 7) Milsana® + *P. flocculosa* (only GR); 8) Milsana® alternated with *P. flocculosa* (only GR), 9) fungicides at recommended rates, on as-needed basis (only GR); 10) TRICHODEX (*T. harzianum* T39, 4 g/l, Mahkteshim Agan LTD) + fungicide (only NL); and 11) Tryptone Soya Broth (OXOID, 3g/l, only GR). Milsana® was used at reduced rates (0.3-0.5%) when it was combined with the microbial biocontrol agents (BCAs). Supplementary *in vivo* bioassays were carried out to test the compatibility of the above-mentioned BCAs with Milsana®. Leaf disks were sprayed either with Milsana®(0.1-1%), *B. brevis* WT, *P. flocculosa* or double combinations of the abovementioned agents. After 24-48 hrs., the leaf disks were homogenized and the suspensions obtained were plated on Tyrosine Agar or PDA amended with 0.2% chloramphenicol (3 x12 discs/treatment). The plates were incubated at 37 or 22°C for 3 days. The colonies of *B brevis* or *P. flocculosa* were counted (+ 3 d) and this value was used to calculate the concentration of cfu/ml.

Powdery mildew was assessed on all leaves of 3-5 plants per plot by estimating the % diseased leaf area. The average severity was calculated for each plant and the results were averaged per plot. *B. cinerea* severity was assessed by counting and removing infected fruits, assessing the number of stem lesions per plant and recording dead plants on five plants per plot. The number and weight of harvested fruits was recorded and specified under first and second class quality (only in NL). The Area Under the Disease Progress Curve (AUDPC) was calculated for powdery mildew and *Botrytis* stem lesions. The average severity for each assessment date, AUDPC variables, total yield (weight) and percentage first class quality yield were analysed with Analysis of Variance, followed by Fisher's protected LSD or Tukey's test. The numbers of cfu/mlof both BCAs were also subjected to ANOVA.

Results and discussion

In Greece, all treatments with Milsana® (single or mixed) reduced powdery mildew severity compared to the control (Table 1). Adding or alternating biocontrol agents did not improve the efficacy of Milsana®. Milsana® applied either as stand-alone treatment or in combination with *P. flocculosa* significantly increased the number and/or weight of harvested fruits (Table 1). Viability of *B. brevis* WT was not affected by Milsana® (0.1-1%). *P. flocculosa* was affected by Milsana® at concentrations over 0.5 %. Also, *B. brevis* WT had a toxic effect on *P. flocculosa* populations (data not shown). In the Netherlands in 2000, powdery mildew was controlled to an extent by *P. flocculosa*. *P. flocculosa* with *B. brevis* WT was significantly better than *P. flocculosa* alone and similar to *B. brevis* WT alone or Milsana® alone. The combination of *B. brevis* WT and Milsana® controlled powdery mildew as good as chemical fungicide plus TRICHODEX (Table 2).

Table 1. Effects of various control treatment on the severity of powdery mildew (in AUDPC) and on cucumber yield in experiments conducted in Greece

	AUDPC (%*days)		Number of fruits/plant		Yield (kg/plant)	
Treatment	2000	2001	2000	2001	2000	2001
Water	2121 a	1960 a	5.3 ab	6.8 a	1.13 ab	1.66 a
Milsana®	1171 c	1664 b	12.2 d	8.3 bc	2.98 d	2.09 ab
B. brevis WT	2019 a		7.2 abc		1.59 abc	
P. flocculosa	2164 a	1921 a	4.9 a	7.3 ab	1.01 a	1.78 a
B. brevis + *P. flocculosa*	1891 a		7.6 bc		1.74 bc	
Milsana® + *B. brevis*	1518 bc		6.4 abc		1.42 abc	
Milsana®+ *P. flocculosa*	1505 bc	1675 b	8.5 c	9.5 c	1.97 c	2.31 b
Milsana® / *P. flocculosa*	1851 ab		5.7 ab		1.22 ab	
Fungicides	428 d	889 c	11.3 d	10.9 d	2.98 d	2.90 c
Tryptone Soya Broth	2120 a		5.3 ab		1.21 ab	

Numbers within a column followed by a common letter are not significantly different at $P \leq 0.05$.

Table 2. Effects of various control treatment on the severity of powdery mildew (in AUDPC), *B. cinerea* (AUDPC's for stem lesions and dead plants) and on cucumber yield in an experiment conducted in the Netherlands, 2000

Treatment	Powdery mildew (AUDPC, %*days)	*B. cinerea* (AUDPC, lesion*days)	*B. cinerea* (AUDPC, dead plants*days)	Total yield (kg/plant)
Water	1249 a	22.9 ab	31.5 a	2.75 a
B. brevis WT	639 c	10.1 b	23.6 a	2.93 a
S. flocculosa	864 b	23.8 ab	19.3 ab	2.85 a
Milsana® 0.5%	602 c	36.7 a	34.1 a	3.31 b
B. brevis+ Milsana 0.5%	377 d	15.9 b	5.2 b	3.69 b
P. flocculosa+*B.brevis* WT	677 c	13.7 b	21.9 a	2.84 a
Fungicide+TRICHODEX	396 d	20.3 b	19.3 ab	3.60 b

Numbers within a column followed by a common letter are not significantly different at $P \leq 0.05$.

In spring 2001 powdery mildew was controlled equally well by all treatments and significantly better by the combination of fungicide and Trichodex (Table 3). In 2000 and spring 2001 (NL), *B. cinerea* on stems was not controlled adequately by any of the treatments (Tables 2 and 3). In autumn 2001, *Botrytis* on fruits was not controlled by any of the treatments, but stem lesions were reduced by *B. brevis* WT and by the combination of *B. brevis* + Milsana®. The number of dead plants in 2000 (Table 2) and autumn 2001 (not shown) was significantly reduced only by *B. brevis* WT + Milsana®. Compared to the control with water, yield was significantly higher in the treatments with Milsana®, *B. brevis* WT + Milsana®, and fungicides + TRICHODEX in 2000 (Table 2), by *P. flocculosa* and fungicide + TRICHODEX in spring 2001 (Table 3) and by Milsana®, *B. brevis* WT, *B. brevis* WT + Milsana®, and fungicides + TRICHODEX in autumn 2001 (data not shown). Differences in the quality of the fruits were insignificant

Table 3. Effects of various control treatment on the severity of powdery mildew (in AUDPC), *B. cinerea* (AUDPC for stem lesions and number of infected fruits per plot) and on cucumber yield in an experiment conducted in the Netherlands, spring 2001

Treatment	Powdery mildew (AUDPC, %*days)	*B. cinerea*		Total yield (kg/plant)
		(AUDPC, lesion*days)	Infected fruits/plot	
Water	791 a	16.6 a	3.5 a	13.58 a
Milsana® 0.3%	264 b	8.8 abc	5.3 a	14.56 abc
Milsana® 1%	184 b	8.8 abc	4.0 a	14.63 abc
P. flocculosa	199 b	10.5 ab	3.5 a	15.04 bc
B.brevis	253 b	1.8 c	3.5 a	13.94 ab
B.brevis+ Milsana® 0.3%	200 b	3.5 bc	1.8 a	13.59 a
Fungicide+TRICHODEX	12 c	8.8 abc	1.5 a	15 41 c

Numbers within a column followed by a common letter are not significantly different at $P \leq 0.05$.

In conclusion, Milsana® gave good protection against powdery mildew in most trials, while *B. brevis* WT and *P. flocculosa* were most effective under Dutch conditions. Control of grey mould stem lesions should be further improved, as combined methods did not enhance control. However, the number of dead plants due to *Botrytis* was decreased by the combination of *B. brevis* + Milsana® indicating that the combination of control agents can increase efficacy against a disease and thus increase yield. Effects on yield could not always be explained completely by the extent to which the diseases were controlled indicating that some treatments may either affect yield directly or induce tolerance in the plant. For implementation of these combinations in practice, other parameters like cost-effectiveness need to be taken into account.

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Efficacy of Milsana® (VP 1999), a formulated plant extract from *Reynoutria sachalinensis*, against powdery mildew of tomato (*Leveillula taurica*)

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Abstract: Milsana® (VP 1999), a formulated extract from the plant *Reynoutria sachalinensis*, was tested against tomato powdery mildew (*Leveillula taurica*). Experiments were carried out in a growth chamber and in commercial greenhouses. In the growth chamber, Milsana® at rates of 1.0%, 0.5%, 0.25% and 0.12% was applied one day and just before artificial inoculation. A preliminary trial and two large scale experiments were conducted in greenhouses. In the preliminary trial Milsana® 0.5%, pyrazophos 0.3 ml/l and water control were tested. In both large scale experiments Milsana® at several rates, water control and fungicides were tested. The efficacy of Milsana varied among the different experiments. All Milsana® rates ≥0.5% were effective. Disease reduction was 35.0% in the preliminary trial and 22.0% and 64.0% in the first and second large-scale greenhouse experiments respectively. Disease reduction by Milsana® (64.8%) and fungicides (92.0%) did not increase yield significantly in the second large-scale experiment.

Key words: Biological control, tomato diseases, *Oidiopsis taurica*

Introduction

Tomato powdery mildew (*Leveillula taurica*), is a severe disease endemic to the Mediterranean basin but may also develop elsewhere. Resistant cultivars and biocontrol means effective against this disease are not available (Belanger et al., 1998). Growers rely on fungicides for protection but often they are not adequately effective (Palti, 1988). Recently, extract of the plant *Reynoutria sachalinensis* (Milsana®) has been reported to be effective against other powdery mildews (Konstantinidou-Doltsinis & Schmitt, 1998; Konstantinidou-Doltsinis et al., 2001). In this paper data on the efficacy of Milsana® against tomato powdery mildew are reported.

Materials and Methods

Growth chamber trial on young tomato plants

Milsana® rate (1.0, 0.5, 0.25 and 0.12% and water control) and time of application (one day and just before artificial inoculation) were tested. Five, four leaf stage, tomato plants per treatment were sprayed to run-off. Plants were inoculated by dusting infected tomato leaves

over them; the plants were incubated for 24 hrs. in a growth chamber at 23°C and 70% RH (60% RH afterwards),12/12 h photoperiod and 10.000 lux light intensity. Records of the percent infected leaf area were taken from the two middle leaves of each plant, 14 days after inoculation.

Greenhouse experiments

Initially, a preliminary trial was carried out in which Milsana® 0.5%, pyrazophos 0.3 ml/l and water control were tested. Two large-scale experiments followed. The first was carried in the farm of TEI in Heraklio (Crete). Treatments consisted of Milsana® (at rates of 1.0, 0.7, 0.5 and 0.3%), penconazole 0.15 ml/l, and water control. The second was carried out in the farm of the Institute of Plant Protection in Patras (Greek mainland) and included Milsana® (at 0.5 and 0.2%), fungicides (myclobutanyl 0.4 ml/l, penconazole 0.15 ml/l and triforine 1.5 ml/l, in alternation) and water control.

In all greenhouse experiments treatments were arranged in a randomized block design; There were two replicates in the preliminary trial and four replicates in the first or and second large scale experiments, respectively. Tomato cv "Manthos" was used in all experiments. Applications started before any disease symptom had observed. Milsana® and water control were applied weekly, whereas the fungicides were applied at 10-day intervals. Records of percent infected leaf area were taken at 7-day intervals and the mean infected leaf area per plant was calculated. In the second large scale experiment the number and fresh weight of fruits per plant was recorded at 7-day intervals and the cumulative number and fresh weight of harvested fruits per plant was calculated. For greenhouse experiments, the disease progress curve for severity was plotted for each treatment. For the two large-scale experiments, the Area Under Disease Progress Curve (AUDPC) was calculated for each plot. Data were subjected to ANOVA and the means were compared by Duncan's multiple range test.

Results

Growth chamber trial on young tomato plants

As shown in Table 1, Milsana® was more effective when applied one hour before inoculation (90% reduction) than when applied one day before (75% reduction). In both cases rates 1.0%, 0.5% and 0.25% significantly reduced infection compared to the control.

Table 1. Effectiveness of different rates of Milsana® (VP1999), applied at the day of inoculation (0 day) or one day before (-1 day), against *L. taurica* on young tomato plants

Treatment	Means of infected leaf area (%)	
	0 day	-1 day
Milsana® 1.0%	0.8 a	8.2 a
Milsana® 0.5 %	2.2 ab	7.8 a
Milsana® 0.25 %	5.0 b	15.0 ab
Milsana® 0.12%	6.0 b	23.0 bc
Control	21.0 c	31.0 c

Statistical analysis was performed by using the SQRT values of disease severity. Numbers with the same letter within the same column are not significantly different ($P \leq 0.05$).

Greenhouse experiments

In the preliminary greenhouse trial, Milsana® reduced infection by 35.0% and the fungicide by 62.5% compared to the control (32.0% infection) (data not shown). Data from the first and second large-scale experiments are shown in Figure 1 and Table 2.

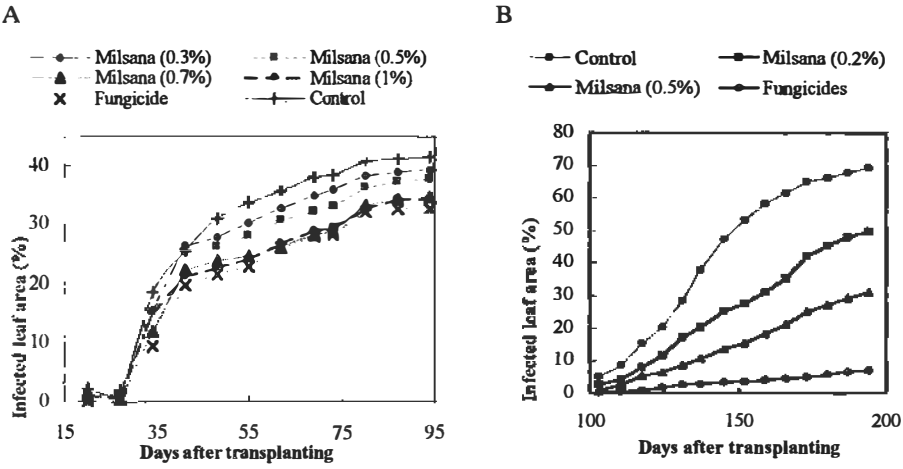

Figure 1. Disease progress on tomato plants in different treatments in A. 1st large-scale experiment and B. 2nd large-scale experiment

Table 2. Effects of Milsana® rate (VP1999) and fungicides on tomato powdery mildew in two greenhouse experiments. Powdery mildew severity is expressed in terms of the Area Under the Disease Progress Curves (AUDPC)

Treatment	1st exp.	2nd exp.
Control	2290 a	3920 a
Milsana® 0.2%		2365 b
Milsana® 0.3%	2116 ab	
Milsana® 0.5%	1995 bc	1389 c
Milsana® 0.7%	1759 d	
Milsana® 1.0%	1779 cd	
Fungicide (s)	1662 d	311 d

Means with the same letter within the same column are not significantly different ($P \leq 0.05$).

Results show that Milsana® at rates of 1.0, 0.7 and 0.5% and the fungicide penconazole significantly reduced disease level compared to the control in the first large scale experiment. Milsana at 1.0 and 0. 7%was equally effective to penconazole. In the second large scale experiment all Milsana® rates and fungicides significantly reduced disease level compared to

the control. Fungicides were significantly better than thel Milsana rates used and Milsana at 0.5% was significantly better than Milsana 0.2%. No significant differences were obtained in terms of yield (kg or number of fruits per plant) among treatments (data not shown).

Discussion

The efficacy of Milsana® varied. In the preliminary trial Milsana® (0.5%) reduced disease by 35.0%. In the first and second large scale experiments Milsana® (0.5%) reduced disease by 23.2% and 69.5% respectively. The low efficacy of Milsana in the first compared to the second experiment may be due to the rapid progress of the disease in the former. In the same experiment, the fungicide penconazole was also not as effective as expected probably due to the predomination of resistant strains. Data from the second experiment indicate that Milsana® efficacy increases as rates increase up to 0.7%. Despite the variability in Milsana efficacy in the above experiments, it is a promising alternative control measure for *L. taurica*. Its lower efficacy in the growth chamber experiment, when applied one day before artificial inoculation compared to its application on the same date, may indicate that frequent applications at lower rates could improve its effectiveness.

In the second experiment, disease reduction by Milsana® or fungicides (64.8 and 92.0%, respectively) did not increase yield. Data reported by other researchers do not always support these results (Palti, 1988; Thomson & Jones, 1981). However, infection started 40 days after the first harvest. At that time one third of the total yield had already been harvested and a high proportion of fruits were fully developed. Moreover, it is assumed that the undetermined type of the cultivars which are grown in greenhouses, such as cv 'Manthos' suffer less than the determined type of cultivars that are mostly grown in the open field. The former continually produce new leaves which might replace the photosynthetic area lost due to the infection.

Acknowledgments

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Effectiveness of natural compounds in the suppression of the powdery mildew fungi *Sphaerotheca fusca* and *Uncinula necator*

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Abstract: The effectiveness of natural substances against *Sphaerotheca fusca* (syn. *S. fuliginea*) and *Uncinula necator* was tested in greenhouse and field trials. In greenhouse, fresh or dried milk (10%) reduced infections on artificially inoculated zucchini squash and cucumber plants over 90% as compared to the untreated control. Powdery mildew severity was significantly reduced also by pinolene (1%), calcium chloride (2%), tripotassium phosphate (1%) and a mixture of mineral oil (1%) with either sodium bicarbonate or sodium silicate (0.5%). In the field, the same compounds yielded a satisfactory control of powdery mildew on zucchini and melon adaxial leaf surface, but resulted poorly effective in controlling infections on stems and on abaxial leaf surface, especially under high disease pressure. In the greenhouse, mineral oil (1%) and plant-resistance inducer acibenzolar-S-methyl (0.005%) proved effective when sprayed preventively, 3-4 days before artificial inoculation. Potassium phosphate dibasic, sodium silicate, lactic acid, azadiractina, calcium nitrate and potassium permanganate were poorly effective when applied at non phytotoxic concentrations. Calcium carbonate, calcium sulphate, trehalose, acetic acid, various types of chitosan, potassium chloride, potassium phosphate monobasic and salicylic acid were ineffective against *S. fusca*. In field trials carried out on table-grape vineyards, pinolene (1%), dried milk (10%), sodium bicarbonate (0,5%), and its mixture with mineral oil (1%), resulted in interesting levels of protection against *U. necator*, but often showed negative side effects on the aesthetical aspect of bunches.

Key words: Powdery mildew, natural substances, cucurbits, grapevine

Introduction

Powdery mildews on cucurbits [*Sphaerotheca fusca* (Fr.) S. Blumer; syn. *S. fuliginea* (Schlecht. *ex* Fr). Poll.] and grapevine [*Uncinula necator* (Schw.) Burr.] are very common and severe diseases in the hot and dry climate of the Mediterranean countries. *Cucurbitaceae* represent one of the most important Italian vegetable crops grown in particular in Central and Southern Italy (Sicily, Latium and Apulia). Melon (*Cucumis melo* L.), watermelon [*Citrullus lanatus* (Thunb) Matsum *et* Nakai], courgette (*Cucurbita pepo* L.) and cucumber (*Cucumis sativus* L.) are the prevalent species being cultivated on a surface of 21,400, 14,200, 12,200 and 1,500 ha, respectively. The great economic relevance of grapevine is clear since it is grown on an area of 892,000 ha (Istat, 2001).

Heavy yield losses are caused by powdery mildew epidemics spreading every year in the areas in which these crops are intensively grown. Many fungicides are available for the control of the disease on these crops. However, at the present, an increasing concern about the use of chemicals in agriculture is evident and people look to more consumer-and

environment-friendly alternatives, including natural substances. A particular worry is related to crops such as courgette and cucumber, requiring repeated sprays during the long harvesting time. This paper reports the results of trials on the effectiveness of natural compounds in the control of powdery mildew in cucurbits and grapes.

Material and methods

Zucchini squash (cv. Striato pugliese) and cucumber plants (cv. Mezzo lungo di Polignano) were grown individually in plastic pots (10 cm in diameter) in a glasshouse at 20-24°C and RH 70-80%, in daylight. Four-replicated plants, maintained at the two-three leaf stage by pruning, were used for each treatment. The plants were artificially inoculated with *S. fusca* by spraying a suspension containing about $5 \cdot 10^5$ fresh conidia/ml. The tested compounds reported in Table 1 included several natural substances allowed in organic agriculture and the chemical substance based on acibenzolar-S-methyl (Bion or Actigard, 50WG, Syngenta), a well-known inducer of plant defence mechanisms (Oostendorp et al., 2001). The compounds were applied either 3 days before or 4 days after artificial inoculation with the pathogen, in order to evaluate their preventive and curative actions. Untreated control plants were sprayed only with water. Numbers and sizes of fungal colonies were assessed on 10-cm^2 of the surface of each leaf, 11 days after inoculation.

During the summer, natural substances were tested in field trials on melon (cv. Barattiere) and table grape plants (*Vitis vinifera* L., cv. Italia) under natural occurrence conditions of powdery mildew infections. All experiments were laid out in complete randomised blocks design with four replicates. Plots consisted of up to 8-10 plants. Spray schedules started before the appearance of the first disease symptoms and sprays were repeated weekly. The severity of powdery mildew symptoms was periodically recorded in order to evaluate the percentage of infected organs, infected leaf surface or infected berries in bunches.

All data, previously transformed in angular values according to Bliss when appropriate, were submitted to analysis of variance and mean values were separated by Duncan's Multiple Range Test.

Results

The tested compounds were grouped in three efficacy groups according to the reduction of symptoms severity as compared to the untreated control; the groups were: effective (more than 70% reduction), moderately effective (40 to 70% reduction) and ineffective (less than 40% reduction) (Table 1).

A good control of both the powdery mildew fungi was achieved in glasshouse as well as in field by fresh or dried milk (10%), which reduced infections over 90%. Lower milk concentrations and skimmed milk were less effective. Sprays carried out after the artificial inoculation, but before symptoms appearance, showed the highest effectiveness. Among the other tested compounds, pinolene, calcium chloride, tripotassium phosphate and a mixture of mineral oil with sodium bicarbonate or sodium silicate significantly reduced the severity of *S. fusca* on leaves. These compounds showed the highest effectiveness when applied 3-4 days after the artificial inoculation of *S. fusca* on potted cucurbit plants in glasshouse. In the field, the same compounds resulted in a satisfactory control of powdery mildew on the upper leaf surface of cucurbits, but they showed poor effectiveness against infections on stems and lower leaf surface, especially under high disease pressure (usually in July-August).

Mineral oil and acibenzolar-S-methyl were effective when applied preventively, 3-4 days before artificial inoculation of *S. fusca*; this confirms these substances acting as inducers of

disease resistance in plants. Moreover, acibenzolar-S-methyl allowed a good disease control when treatments were repeated at one-week intervals, while the concentration found effective in single treatments (0.05%) resulted to be phytotoxic and responsible for plant disorders.

Potassium phosphate dibasic, sodium silicate, lactic acid, azadiractina, calcium nitrate and potassium permanganate proved poorly effective at non phytotoxic concentration while calcium carbonate, calcium sulphate, trehalose, acetic acid, various types of chitosan, potassium chloride, potassium phosphate monobasic and salicylic acid were ineffective against *S. fusca*.

Table 1. Effectiveness of natural substances tested in greenhouse and field trials against *Sphaerotheca fusca* and *Uncinula necator* [a]

Compound	Rate (%)	Trials		
		Glasshouse	Field	
		Cucurbits	Cucurbits	Grapevine
Pinolene	1	+++	+++	+++
Calcium chloride ($CaCl_2$)	1.5-2	+++		
Fresh milk	100	+++		
Dried milk	10	+++	+++	+++
Tripotassium phosphate (K_3PO_4)	1	+++		
Acibenzolar-S- methyl (Bion)	0.005	+++		
Mineral oil	1	+++	+++	
Sodium bicarbonate ($NaHCO_3$)	0.5	+++	+++	+++
Mineral oil + sodium bicarbonate	1+0.5	+++	+++	+++
Mineral oil + sodium silicate	1+0.5	+++	+++	
Potassium phosphate dibasic (K_2HPO_4)	1	++	++	
Sodium silicate ($Na_2O.2SiO_2$)	0.5	++		
Lactic acid ($CH_3CHOHCOOH$)	1	++		
Azadiractina	0.5–0.15	++		
Calcium nitrate [$Ca(NO_3)_2$]	1	++		
Potassium permanganate ($KMnO_4$)	1	++		
Calcium carbonate ($CaCO_3$)	1	+		
Calcium sulphate ($CaSO_4$)	1	+		
Trehalose ($C_{12}H_{22}O_{11}.2H_2O$)	1	+		
Acetic acid (CH_3COOH)	0.5	+		
Chitosan	0.5	+		
Potassium chloride (KCl)	1	+		
Potassium phosphate monobasic (KH_2PO_4)	1	+		
Salicylic acid (HOC_6H_4COOH)	0.5	+		

[a] +++ effective; ++ moderately effective; + ineffective.

In the field trials, pinolene, dried milk, sodium bicarbonate, and its mixture with mineral oil, proved highly effective against *U. necator*, but they had some negative side effects on bunches, such as wax removal, dirtiness and bad smell; therefore it is advisable to use these compounds on table grapes carefully.

Discussion

The results discussed in this paper confirm and study in detail some previous observations on the effectiveness of natural substances (Casulli et al., 2000; *Santomauro et al.*, 2001) showing that such compounds, in agreement with Di Giovannantonio et al. (1997) and La Torre et al. (1999), are effective in the early stages of infection or under low disease pressure. Moreover, natural substances are more effective when applied repeatedly at short time intervals. Their effectiveness against powdery mildews proved to be influenced by temperature, time of application and in particular uniformity of distribution on plants. In fact, field trials showed that one of their most important limitations is the lack of control of powdery mildew on the lower surface of cucurbits leaves.

Natural substances that are really effective against powdery mildews may be useful to reduce the use of chemicals under conditions of low disease pressure, besides in biological or organic farms also in conventional agriculture. Therefore, it is fundamental to take into account the increasing demands of consumer-and environment-friendly integrated crop protection management. Some crops, such as cucurbits, would benefit greatly from the usage of natural substances since they require treatments even during the rather long period in which fruits are continuously harvested.

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Feeding of mycophagous ladybird, *Psyllobora bisoctonotata* (Muls.), on powdery mildew infested plants

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Abstract: Ladybird, *Psyllobora bisoctonotata* (Muls.) is a mycopredator of powdery mildew. It has been recorded on some species in powdery mildew genera. This mycopredator first came to our attention during recent survey conducted in Samandağ and Arsuz districts where vegetable are produced extensively. In these areas powdery mildew infestation is suppressed by natural infectation of the mycopredator. High proportion of population of the coccinellid *P. bisoctonotata* (Muls.) was found on the okra leaves infected by powdery mildew agent, *Erysiphae cichoracearum*. In these regions, okra is sustained commercially in small fields with low inputs. We were interested to find whether this insect could be used as a potential biocontrol agent of powdery mildew. When the insect was reared in the laboratory and released on powdery mildew infected okra leaves, both larval and adult stages fed on the fungal organs. Insect-released leaves were cleared and stained with lactophenol-tryphan blue solution and examined under light microscope. Microscopical observations on these leaves revealed that both stages of the insect ate vegetative (mycelium) and reproductive structures such as conidia and conidiophores produced by the fungus on the surface of leaves. In order to determine the efficacy of feeding on inoculum source, a certain part of insect-fed leaves (1 cm^2) was excised and conidia were eluted from the leaves and counted on a haemocytometer slide. The number of conidia on insect-fed leaves was reduced by 92% as compared to the non-feeding side of the infected leaf. Feeding area on sporulating leaves by different insect stages was also evaluated daily for a week. Third and forth larval stages were found to be the most efficient feeding stages and these were followed by adult, 2 nd and 1 st stages respectively. Specificity of the insect against a number of powdery mildew species was also studied *in vitro* on detached leaves infected by powdery mildew agents. Apart from okra, the insect was also observed feeding on leaves of several plants such as mulberry, plane trees, English laurel, cucumber, pepper, tomato and weeds infested with different powdery mildew species belonging to *Phyllactinia, Microsphaera, Sphaerotheca* and *Leveillula* genera.

Key words: Erysiphae, okra, *Psyllobora*, powdery mildew, biological control

Introduction

Fungal diseases are the major and frequently reported causal agents reducing field and greenhouse vegetable production around the world. Powdery mildew caused by members of Erysiphae genera are disease of numerous field and greenhouse crops and has a world-wide distribution. *Erysiphae cichoracearum* is the causal agent of powdery mildew of vegetable such as cucurbits, okra, ornamentals and non-cultivated plants such as weeds. The disease occurs in Europe on about 230 plant species (Smith et al., 1988). Average yield losses of 20-30% have been attributed to the disease in some countries (Dixon, 1978). Despite extensive studies on their epidemiology and pathogenesis, the disease they cause remains among the most important problem facing plant pathology worldwide. In greenhouses, powdery mildew diseases are particularly aggressive because constant favorable environmental conditions

accelerate their development. If disease incidence is high the fungus can cause extensive defoliation leading to a reduction of economic crop yield. Control of the disease relies mainly on multiple applications of chemical fungicides. Combinations of high inoculum pressure, glasshouse conditions that favour fungal growth, and frequent pesticides application, have resulted in emergence of resistant pathogen strains. The risk of fungus resistance and the public concern about the reduction of the use of pesticides on products ready to eat, forced researchers to search alternative control ways.

In recent years, researchers have proposed new avenues to facilitate the development of safer technologies for disease control and environment. Among the proposed alternatives, biocontrol has been drawn attractions. Classical biocontrol of plant disease using antagonistic microorganisms may offer a sustainable and environmentally-friendly solution. This biocontrol approach involves the introduction and establishment of antagonistic microorganisms that infect the target disease agent in their native environment. In addition to antagonistic microorganisms, certain insects may be also used as a biocontrol agent (BCA) of plant diseases. Okra (*Hibiscus esculantum* L), which is consumed as a fresh vegetable, is sustained commercially with low inputs by several small to medium size growers throughout the Hatay province (Anonymous, 2000). Powdery mildew is the major disease on okra plant facing farmers in the region. Fungicide application against to the disease is not common, since crop harvest is made daily. Recently, we have noticed high population of ladybird, *Psyllobora bisoctonatata* on the okra leaves infested by powdery mildew. We were interested whether this insect could be used as potential BCA of powdery mildew. This paper reports on mycophagous ladybird, *P. bisoctonotata* (Muls.) that is currently under investigation as a potential biocontrol agent for the powdery mildew agent, *E. cichoracearum*.

Material and methods

Seeds of okra were obtained from the natural population of plants in Samandağ from which an isolate of *E. cichoracearum* was collected. Healthy plants were routinely grown in 18-cm pots in growth room with 12 h photoperiod, a light intensity of 40 W/m^2 and a temperature of 25±2°C. Since *E. cichoracearum* is an obligate parasite, the fungal isolate was maintained on susceptible okra leaves. The plants were inoculated with conidia of bulk isolate of *E. cichoracearum* by shaking heavily infected plants over healthy leaves. The inoculated plants were kept in growth room at 25±2°C day/light temperature until use. Adults of *Psyllobora*, collected from the natural population in Samandağ, were identified as *P. bisoctonotata* by Prof. Dr. Nedim Uygun, Department of Plant Protection, University of Cukurova, Adana, Turkey. This insect was reared and maintained on powdery mildew sporulating okra leaves throughout the experiment. The method employed was a modification of that used by Yigit (1992). For determination of daily consumption of larvae and adults, heavily sporulating okra leaves were detached from the plant and placed in plastic pots, which were filled with water. Newly hatched larvae and adults were separately transferred on upper surface of heavily sporulating okra leaves which were confined in the sleeved cages. Feeding behaviour and the amount of larvae consumption were monitored daily until the larvae had reached the pupal stage (n=15). Daily adult consumption was, similarly, recorded for 8 days (n=15). Infested leaves were changed every second day and larvae or adults were transferred to the new fresh leaves. In order to determine the effect of feeding on fungal inoculum, spore concentration were calculated from insect released and fed tissues by cutting three leaf pieces (1 cm^2) from the region of feeding. Excised discs were suspended in distilled water and fungal conidia were counted on haemocytometer. Microscopical observation was conducted to reveal which part of fungal structure is consumed by the insect. Insect-fed leaf parts were detached from plant,

cleared and stained with lactophenol-tryphan blue solution, and examined under light microscope. Specificity of the insect against a number of powdery mildew species was also studied *in vitro* on detached leaves infected by powdery mildew agents.

Results and discussion

During extensive field surveys in Samandağ and Arsuz in September 2001, *P. bisoctonotata* was found to feeding on the okra leaves infested by *E. cichoracearum*. Proportion of insect population and activities in powdery mildew infested fields was considerably higher than those fields where the disease was either absent or it was prevailing at low intensity. It was found that both, the larval and the adult stages fed with the fungus. Microscopic studies further revealed that both stages feed on the leaf surface until entire fungal structures, such as extracellular mycelium, conidiophores, and conidia produced by the fungus on the surfaces of the leave, were consumed. After feeding, pieces of the larval feces were collected and placed on the healthy leaf surface. No fungal growth was observed in repeated experiments. Results suggest that all fungal structures that passed through the larval digestive tract were completely digested. Among the different stages of the insect, third and fourth larval stages were the most efficient feeding stages, followed by adult, second and the first stages, respectively. Each individual larvae (from the 1st to the 4th stage) or adult consumed the approximately 12,3 cm^2 powdery mildew infested area, 8 days after releasing on the leaves. The fungal inoculum source on insect-fed leaves was reduced by average 92% as compared with the control.

Several organisms which feed on or parasitize powdery mildew have been evaluated as possible BCA. Fungi such as *Pseudozyma flocculosa* (syn. *Stephanoascus flocculosus*) and closely related species *P. rugulosa* have been reported as natural BCA of powdery mildew agent *Sphaerotecha fuliginea* (Traquair et al., 1988; Jarvis et al., 1989) and results have prompted interest in the development of *P. flocculosa* as a biofungicide (Paulitz & Belanger, 2001). In addition to fungi, several Arthropods and nematodes have been also reported to have antagonistic potential against plant pathogenic fungi such as *Sclerotinia* and *Pythium* (Anas & Reeleder, 1987; Jarvis et al., 1993; Gracia-Gorza et al., 1997). The Coccinellidae (Coleoptera) is perhaps the best-known family among our native beetles. These, and many other ladybirds, are voracious predators, feeding in both larval and adult stages on aphids, coccids (scale insects), mealybugs, whiteflies and, mites (Borror et al., 1992). Some species of ladybirds, belonging to *Psyllobora* genus, are however non-predatory. Different species of *Psyllobora* have been previously reported to feed on different powdery mildew agents. *Psyllobora cincta* was found feeding by *Oidium lini* on flax, *P. bicongregata* by *E. cichoracearum* on squash, *P. nana* by *E. polygoni* on sunflower (Prasad & Rai, 1988; Cruz et al, 1990; Bado & Rodriguez, 1998). *P. bisoctonotata* was previously reported on citrus (Uygun, 1981) in Turkey. This is, however, the first report of this coccinellid as a BCA of powdery mildew in Turkey. Our study showed that *P. bisoctonotata* has broad host range. Apart from okra, both adults and larvae were also observed feeding on leaves of several plants such as mulberry, plane trees, English laurel, cucumber, pepper, tomato and weeds infested with different powdery mildew agents such as *Phyllactinia guttata, Microsphaera platani, Microsphaera euonymi-japonici, Sphaerotheca fuliginea, Leveillula taurica*. The yellow and black 22-spot ladybird, *P. vigintiduopunctata*, was found to feed on plant leaves infested with different powdery mildew agents (Sadeqhi & Esmailli, 1992; Ratti, 1996). We also found adults of *P. vigintiduopunctata* on okra leaves, but this species was not efficient as *P. bisoctonotata*.

The overall results suggest that *P. bisoctonotata* may provide only partial control of powdery mildew. Integration of this insect with cultural practises and organic pesticide

increases the certain probability of achieving reliable and acceptable control in certain places such as greenhouses.

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Survival and activity of biocontrol yeasts against powdery mildew of cucurbits in the field

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Abstract: In a two-year (2000 and 2001) investigation carried out in melon fields of Southern Italy, the antagonistic activity of the yeasts *Rhodotorula glutinis, Cryptococcus laurentii* and *Aureobasidium pullulans* against powdery mildew of cucurbits (*Sphaerotheca fusca,* syn *S. fuliginea*) was evaluated. In both years, the antagonists reduced disease incidence on leaves significantly showing an activity comparable to that of the fungicide penconazole. In the first year the yeasts were more effective than biofungicide AQ10, while in the second year their activity was similar to that of AQ10. The antagonists showed good survival on leaves surface even if the climate was quite hot and dry with rare rainfalls. Further investigations are necessary in order to optimise the activity of the new proposed biological means for the control of cucurbits powdery mildew.

Key words: powdery mildew, cucurbits, biocontrol, antagonist yeasts

Introduction

Powdery mildew of cucurbits, caused by *Sphaerotheca fusca* Blumer [sin. *S. fuliginea* (Schlecht.: Fr. Pollacci)], is the most common and severe fungal disease of these crops in the Mediterranean areas. Control of the pathogen is currently carried out by several sprays with synthetic fungicides, which cause detrimental effects to the environment as well as risks for resistance development in the fungal populations. These aspects and the increasing development of more sustainable agricultural systems have stimulated the research of suitable alternatives such as biocontrol by antagonistic microorganisms and/or natural compounds (Belanger, 1999; Santomauro et al., 2001). Several microorganisms have been found to be effective against *S. fusca*, but their survival and effectiveness, particularly in open field, are often variable due to the fluctuating climatic conditions; therefore, an antagonist to control efficiently the pathogen must survive at high level of population also under variable climatic conditions.

The aim of this study was to evaluate the antagonistic activity of some selected yeasts against powdery mildew and their survival on melon leaves in open field. The potential antagonists were previously isolated from different crops and then they characterized for activity against other fungal pathogens (Lima et al., 1999a,b).

Materials and methods

Trials were performed in the years 2000 and 2001 on melon cv Madras grown in fields of Southern Italy. The yeasts *Rhodotorula glutinis* (Rg) and *Cryptococcus laurentii* (Cl) and the yeast-like fungus *Aureobasidium pullulans* (Ap) were tested as potential antagonists of *S. fusca*; the yeast Rg was tested only in the year 2001. The yeasts (10^7 cfu/ml) were applied weekly by a motorized sprayer at a volume of 1000 l water/ha). The fungicide penconazole (Topas 10EC®, 25 g/hl), applied at intervals of 14 days, and the biofungicide AQ10® (*Ampelomyces quisqualis*),

applied weekly, were also included as controls. Both potential antagonists and AQ10, before their application, were suspended in a 0.2% (v/v) of a ultra fine mineral oil (UFO®, Intrachem). Treatments started in the 2nd half of June, before appearance of powdery mildew symptoms, and terminated at the end of July, at full development of fruits.

Individual plots were rows with 6 plants, spaced 2 m apart and arranged in a complete randomised block design with 4 replications. Powdery mildew incidence and total yeast population (white yeasts, pink yeasts and yeast-like fungi) were assessed periodically. Disease severity was evaluated on adaxial surface of all leaves using an empirical scale with 5 degrees of disease, ranging from 0 (no infection) to 5 (100% of leaf surface covered by the pathogen).

Data were processed using McKinney disease index and submitted to variance analysis. Mean values of disease incidence were compared using Duncan's multiple range test. Percentages were converted into Bliss angular values (arcsin √%) before analysis.

Yeasts population was assessed on surface of leaves randomly taken weekly from each plot as previously reported for population studies on fruit surfaces (Lima et al., 1999b). Daily temperature and relative humidity were constantly recorded in the field using a thermo-hygrometers (1750B, Salmoiraghi, Milano, Italy). Daily rainfall (mm) was obtained from a local climatologic station.

Results

In 2000, the yeasts Cl and Ap reduced disease incidence by 56 and 49%, respectively; in 2001, Rg, Cl and Ap reduced disease incidence by 44, 35 and 27%, respectively. Yeasts activity was comparable to that of chemical control in both years. With respect to AQ10, the antagonists showed higher activity in the first year, when the disease pressure was higher and an activity similar to AQ10 in the second year (Figure 1).

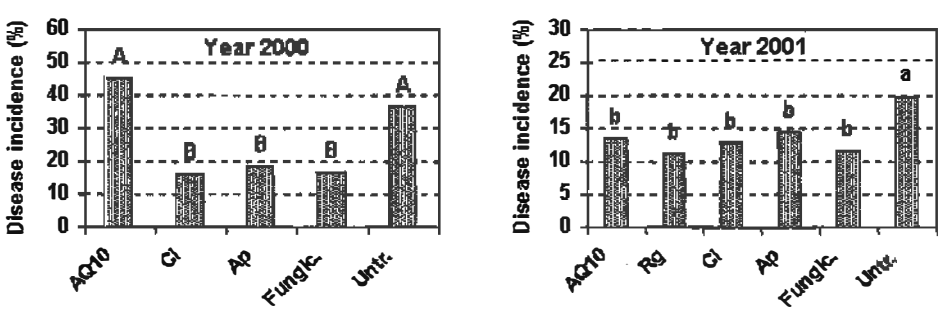

Figure 1. Severity of powdery mildew infections on melon leaves subjected to different treatments in the field: AQ10= *A. quisqualis*; Rg= *R. glutinis*; Cl= C. *laurentii*; Ap= *A. pullulans*; Fungicide= Penconazole; Untr.= Untreated. Values marked by same letters are statistically not different at P=0.05, small letters, and at P=0.01, capital letters, (Duncan's test).

The total yeasts population was significantly higher on leaves treated with the antagonists as compared to leaves not treated with yeasts (untreated, fungicide and AQ10 plots). In 2000, starting from the 6th of July yeast populations increased and reached higher levels as compared with the populations recorded in 2001 (Figure 2).

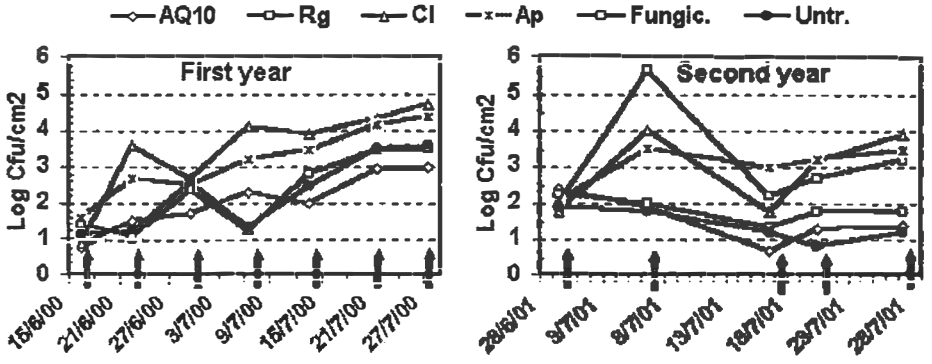

Figure 2. Dynamic of total yeast population (white yeasts + pink yeasts + yeast-like fungi) on melon leaves subjected to different treatments in field: AQ10= *A. quisqualis*; Rg= *R. glutinis*; Cl= C. *laurentii*; Ap= *A. pullulans*; Fungicide= Penconazole; Untr.= Untreated. The arrows indicate application of treatments.

In the first year, when powdery mildew incidence was high observed, the climate was hot and dry; maximal temperature frequently rose above 30°C and relative humidity dropped under 40%. In both years, only 1-2 rainfalls occurred in the second half of July. These few precipitations induced a drastic reduction of maximum temperature and an appreciable increase in relative humidity (Figure 3).

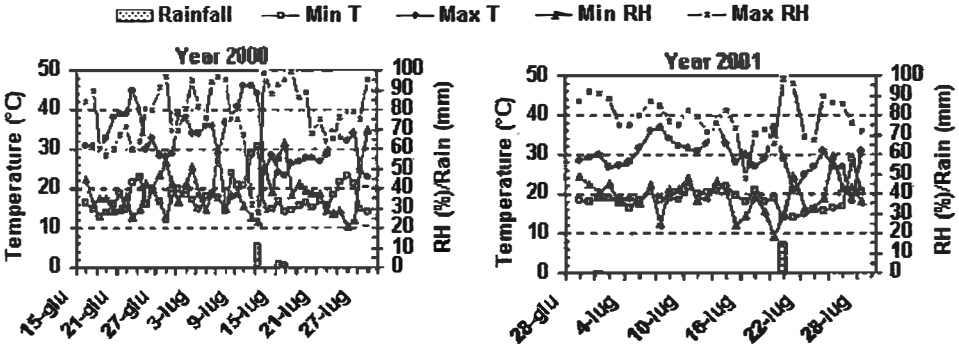

Figure 3. Climatic conditions in melon fields during the periods of the experiments.

Discussion

Yeasts and yeast-like fungi are wide-spread epiphytic microorganisms that are well adapted to phyllosphere and carposphere of herbaceous and woody plants (Andrews, 1992; Blakeman & Fokkema, 1982; Lima et al., 1999a). Their antagonistic activity against necrotrophic fungal pathogens was reported in different investigations (Elad et al., 1994;

Lima et al., 1997, 1999b). However there is a lack of information about their possible activity against biotrophic fungal pathogens.

The tested isolates of Rg, Cl and Ap, expressed an appreciable activity against *S. fusca* in open field under hot and dry climate. This was observed in particular in the first year, when disease pressure was higher. Under these conditions, AQ10 resulted in poor efficacy. The tested yeasts isolates survived on leaf surfaces at appreciable levels even under these conditions. Moreover, following some rainfalls which caused reduction of temperature and an increase in relative humidity, the yeast population reached higher levels. These antagonists are interesting because they may control, besides S. fusca, also other fungal pathogens of cucurbits, such as *Botrytis cinerea* (Elad et al., 1994; Dik et al., 1999).

The results of this study encourage further investigations aimed to optimise the new proposed biocontrol means and to verify if some modes of action already expressed by yeasts against necrotrophic fungi (Castoria et al., 1997; 2001) are also involved against the biotrophic fungus *S. fusca*.

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Biological control of grapevine powdery mildew with Effective Micro-organisms (EM)

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Effective Microorganisms (EM) a mixture containing lactic acid and photosynthetic bacteria and yeast, isolated from the respective ecosystems and not just from single source were tested.
The stock solution of biological preparates EM-1 and EM-5 (commercially produced by "Multicraft", Haiding, Austria) were investigated.

The experiment lasted two consecutive cultivation period and Riesling vine cultivar was used. Totally seven treatments were performed in the year 2000 and 14 in the year 2001. The first was done at the vegetative stage 15 (EL shoot stage scale). EM preparates were examined in concentration 5.0% and 0.2 % respectively. As the reference products combination of fungicides were used.
The efficiency of spraying by EM-1 and EM-5 were 98.91% and 97.64 % in 2000 and 75.23% and 70.20% in 2001 respectively. Results from the two-year field trails showed that the EM preparates could control disease very good when low or medium level of infection appeared but when the infection increased effectiveness subsequently decreased. The number of treatments was directly dependent on rainfall during vegetation.

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Effect of biocontrol antagonists applied in combination with calcium on the control of postharvest diseases in different fruits

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Abstract: The biocontrol efficacy of *Candida guilliermondii*, *Pichia membranefaciens*, *Trichosporon* sp. and *Cryptococcus laurentii*, applied with or without addition of calcium, was determined for the control of postharvest disease in peach, nectarine, apple and grape, respectively. Addition of Ca to the suspensions of *C. guilliermondii* and *P. membranefaciens* resulted in lower disease incidences and smaller lesion sizes in peach and nectarine fruits infected by *Rhizopus stolonifer*, as compared with treating with the yeasts alone. The addition of calcium did not directly increase the population size of the antagonists *in vitro*, but inhibited spore germination and germ tube growth of *R. stolonifer* in PDB. Effect of controlling gray and blue mould rots in apple fruits by *Trichosporon* sp was enhanced when $CaCl_2$ in an aqueous suspension was added, even when the biocontrol agent was applied at a low concentration of 10^5 cfu/ml. On the other hand, addition of calcium did not enhance biocontrol efficacy of *C. laurentii* on postharvest diseases of grapes during storage periods.

Key words: biocontrol efficacy, yeast, calcium, postharvest disease, fruit

Introduction

Biocontrol of postharvest decays of fruits and vegetables has emerged recently as a promising alternative to the use of synthetic fungicides. Treatment of fruit with microbial agents has been demonstrated to be an efficient method for control of several postharvest decays (Canway et al., 1992; Fan & Tian, 2000). Some yeasts and bacteria are reported to reduce effectively various postharvest decays of fruits (Piano et al., 1997; Fan & Tian, 2001). In order to enhance biocontrol efficacy of the antagonists against fungal pathogens, certain strategies, such as adding calcium salts, carbohydrates, amino acids and other nitrogen compounds to biocontrol treatments, are proposed (Janisiewicz et al., 1992).

Many researchers have shown that Ca plays an important role in the inhibition of postharvest decay of fruits (For example, Conway et al., 1992) and in enhancing the efficacy of postharvest biocontrol agents (Wisniewski et al., 1995). The objective of the study was mainly to determine if the biocontrol efficacy of *Candida guilliermondii*, *Pichia membranefaciens*, *Trichosporon* sp. and *Cryptococcus laurentii* is enhanced when applied in combination with Ca, for the control of postharvest diseases in peach, nectarine, apple and grape, respectively.

Materials and methods

Pathogens and antagonists

Penicillium expasum, *Botrytis cinerea* and *Rhizopus stolonifer* were isolated from infected apple and peach fruits. The suspensions of *P. expasum*, *B. cinerea* and *R. stolonifer* were applied the concentrations of 1×10^5, 5×10^4 and 5×10^6 spores/ml, respectively. *C.*

guilliermondii, P. membranefaciens, Trichosporon sp. and *C. laurentii* were isolated from peach and apple fruits. The cell suspensions of *C. guilliermondii* and *P. membranefaciens* were adjusted to various concentrations (5×10^8, 5×10^7, 5×10^6, 5×10^5 and 0 cfu/ml) with or without 2% w/v $CaCl_2$ *in vitro* and *in vivo*. The cell suspensions of *Trichosporon* sp. were made in 2, 1 and 0% $CaCl_2$, then adjusted to concentrations of 10^8, 10^7, 10^6, 10^5 and 0 cfu /ml, respectively. The suspensions of *C. laurentii* were adjusted to a concentration of 10^7 with different concentrations of 0, 0.5, 1 and 2% w/v $CaCl_2$.

Fruits

Peach (*Prunus persica*), nectarine (*P. persica* var. nectarina.), apple (*Malus domestica* Borkh. cv. Fuji) fruits and grapes were used in the experiment. The fruits were wounded, and then a 30 µl drop containing the yeast suspensions and a 20 µl drop containing suspension of the pathogen were simultaneously applied to each wound. The fruits were stored at 25°C with about 95% r.h humidity.

Results and discussion

***Effect of calcium and concentrations of antagonists on pathogen* in vitro**

Calcium and the concentration of yeast suspensions affected spore germination and germ tube growth of *R. stolonifer in vitro* (Figure 1). Germination rate and germ tube length decreased when concentration of yeast cells increased from 0 to 5×10^8 cfu/ml. However, the addition of Ca to the same concentrations of yeast suspensions significantly decreased spore germination rate and germ tube length of the pathogen compared with treatments with antagonists alone after 13 hrs incubation at 25°C. The growth of *R. stolonifer* could be completely inhibited at a concentration of 5×10^8 cfu/ml with or without calcium (Figure 1).

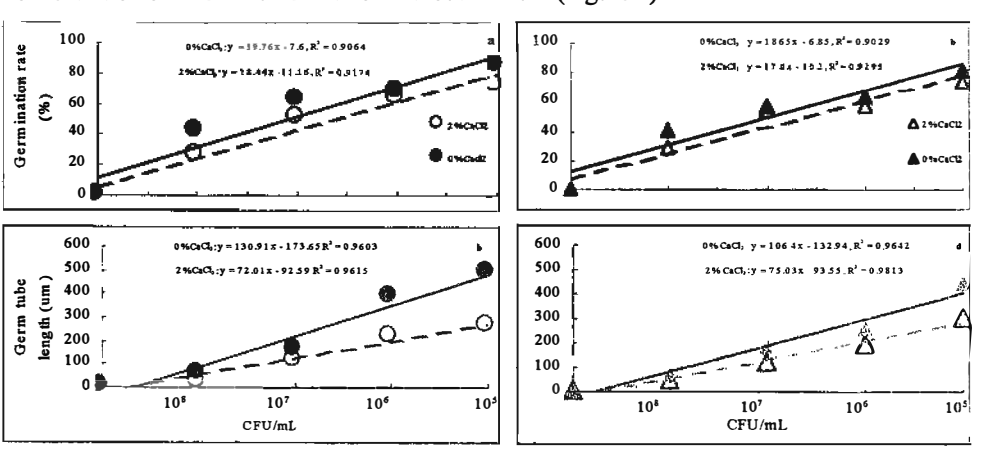

Figure 1. Effects of calcium and different concentrations of *Candida guillermondii* (a, b) and *Pichia membranefaciens* (c, d) cell suspensions on the germination rate and germ tube length of *Rhizopus stolonifer* spores in potato dextrose broth after 13 hrs incubation at 25°C.

Effect of calcium on biocontrol activity of* C. guilliermondii *and* P. membranefaciens *for control rhizopus rot in peach and nectarine fruits

The degree of *R. stolonifer* infection in peach and nectarine fruits decreased with increasing the concentration of yeast antagonists. Combining calcium with *C. guilliermondii* significantly reduced disease incidence and inhibited lesion size of Rhizopus rot in peaches (Figures 2a and b) ($P \leq 0.05$). A similar effect was observed with calcium and *P. membranefaciens* for the control of Rhizopus rot in nectarines (Figures 2c and d). When yeast cell suspensions of *C. guilliermondii* and *P. membranefaciens* were applied at a concentration of 5×10^8 cfu/ml, no infection by *R. stolonifer* was found in peach and nectarine fruits treated with or without the presence of calcium.

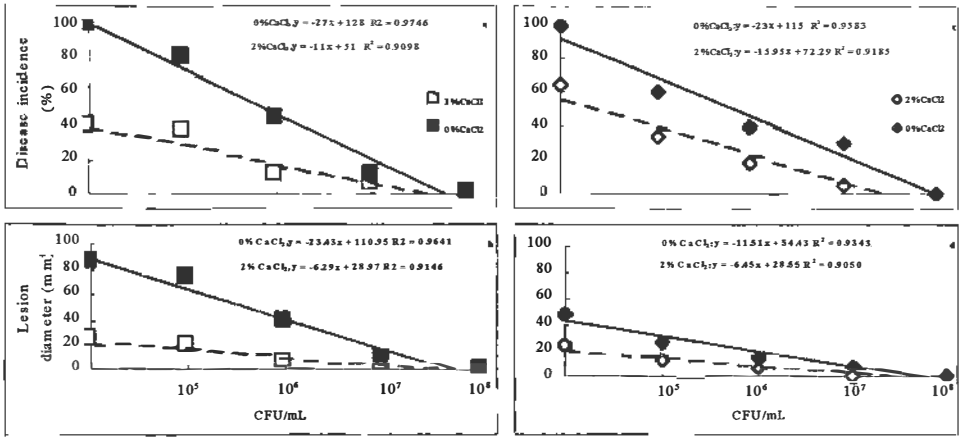

Figure 2. Effects of calcium and cell concentrations of *Candida guilliermondii* (a, b) and *Pichia membranefaciens* (c, d) on disease incidence and lesion development of *Rhizopus stolonifer* in peach (a, b) and nectarine (c, d) fruits stored at 25°C for 3 days

Effect of calcium on biocontrol activity of* Trichosporon *sp. for control blue mold and grey muold in apple fruits

Significant effects of Ca in the inhibition in gray and blue mould rots were achieved in this experiment (Figure 3). Adding $CaCl_2$ to the yeast cell suspensions resulted in lower disease incidence and smaller lesion size. The yeast concentration of 10^8 cfu/ml in 1 and 2% $CaCl_2$ totally inhibited gray and blue mould rots in apple fruits. Meanwhile, higher yeast concentrations in combination with 2% $CaCl_2$ showed the most effective inhibition in infections by *B. cinerea* and *P. expansum* (Figure 3). The yeast concentrations either in 0 or 1-2% $CaCl_2$ also significantly affected the biocontrol efficacy. The lower concentration the antagonist, the higher the disease incidences.

Acknowledgements

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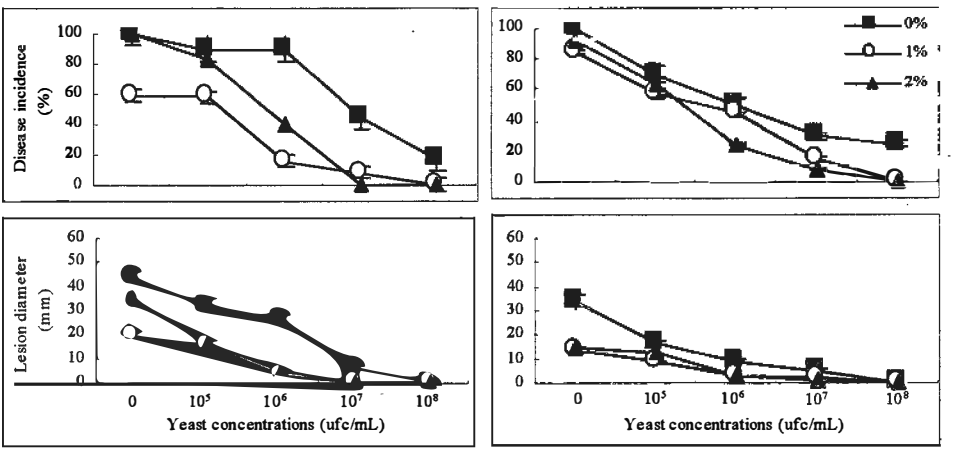

Figure 3. Effects of $CaCl_2$ addition to cell suspensions of *Trichoderma* sp. On disease incidence and lesion development of Botrytis cinerea (left) and *Penicillium expansum* (right) in apple fruits after 7 days at 25°C

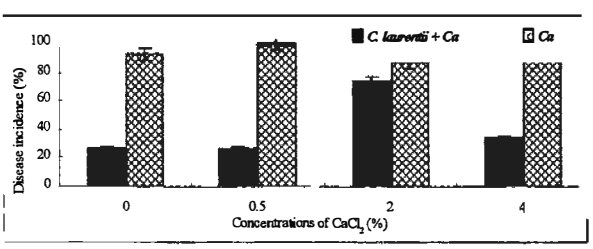

Figure 4. Effects of different concentrations of $CaCl_2$ on biocontrol efficacy of *Ryptococcus laurentii* to control wound infection of grapes after 2 days at 25°C

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Development and commercial testing of the yeast *Metschnikowia fructicola* for the control of pre and postharvest diseases

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Abstract: Pesticide residues on fruits and vegetables are a major concern to consumers and to the fruit and vegetable industry. Development of safer alternatives that are effective and pose less risk to human health and the environment has been one of the major research topics in recent decade. The development of antagonistic microorganisms has been the most studied and substantial progress has been made in this area. Currently, four antagonistic microorganisms, two yeasts; *Candida oleophila* and *Cryptococcus albidus*, and two strains of the bacterium *Pseudomonas syringae* are commercially available under the trade names ASPIRE, YieldPlus, and BIOSAVE-110, respectively. All these products are recommended for use against postharvest diseases of pome and citrus fruit.

The yeast antagonist *Metschnikowia fructicola* was isolated from the surface of table grapes in Israel. This yeast has been tested during the last three years for its biocontrol efficacy against wide range of pre and postharvest diseases of fruits and vegetables. It was fond effective in controlling Aspergillus and Botrytis rots developed on wine grapes in the vineyard. Marked reduction in Botrytis, Aspergillus and Rhizopus decay was also observed on table grapes after storage and shelf-life.

The effect of preharvest application of *M. fructicola* was also tested against preharvest and postharvest rots on strawberry in Turkey and Israel. Over a period of two growing seasons the efficacy of yeast antagonist against preharvest rots was equal to that of chemical control. In an open-field experiment, the yeast was also able to reduce decay incidence to commercially acceptable levels (less than 5%). In storage experiments covering 3 days at 1°C followed by two days of shelf life, the incidence of decayed fruits that were treated with yeast in the field was significantly lower than that of water and chemical control.

Yeast cells were produced by submerged fermentation in an industrial growth medium. The biomass was formulated into Water Dispersible Granules (WDG) that has a shelf life of one year. The product was applied by conventional spraying equipment , and was found compatible with most of the common pesticides. No symptoms of toxicity were associated with the formulated cells in toxicological studies, paving the way to the registration of this safe product as a biofungicide.

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Biocontrol of postharvest fungal pathogens of peaches and apples by *Pantoae agglomerans* strain IC1270

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Abstract: Commercially ripe peaches and apples were wounded and co-inoculated with either conidia of *Monilinia fructicola,* spores of *Penicillium expansum* or sporangiospores of *Rhizopus stolonifer* in combination with a wide-range antagonist of plant-pathogenic fungi *Pantoae agglomerans* strain IC1270 producing a set of secreted chitinases and antibiotic pyrrolnitrin (Prn). Application of strain IC1270 (10^9 cfu/ml) on peaches infected by *M. fructicola* (up to 10^5 conidia/ml) or *R. stolonifer* (up to 10^5 spores/ml) and stored at 20-25°C and RH 90-95% reduced the incidence of the diseases and the size of lesions. The efficiency of biocontrol effect was dependent of the pathogen concentration. Tn5-mutants of IC1270 deficient in chitinolytic activity but producing Prn showed a similar efficiency towards both these pathogens as the parental strain. Moreover, Prn purified from strain IC1270 and co-inoculated with the fungi into the fruit wound completely suppressed Rhizopus rot and reduced the Monilinia rot incidence. The data show the importance of Prn as a key factor of strain IC1270 biocontrol activity in post-harvest storage. In addition strain IC1270 was shown to be highly efficient in control of blue mold disease caused by *P. expansum* on apples.

Keywords: chitinases, pyrrolnitrin, Monilinia brown rot, Penicillium blue mold, Rhizopus rot

Introduction

Rots caused by *Rhizopus stolonifer* (Ehrenb.: Fr.) Lind. and *Monilinia fructicola* (Wint.) Honey and blue mold caused by *Penicillium expansum* Link are among the most destructive postharvest diseases of stone and pome fruits through the world. Chemical treatment is generally an efficient way for controlling plant pathogenes, including those causing postharvest diseases, however development of fungicide-resistant pathogens and public concerns over the presence of chemical residues in food has resulted in cancellation of some of the most effective fungicides. Therefore, research has been focused on the development of management alternatives that are both effective and economically feasible. The biocontrol of postharvest diseases has been widely reported and microbial biocontrol agents (BCA) have shown promise as potential alternatives to fungicides. Several yeast and bacteria were found promising for biocontrol of postarvest diseases caused by *Rhizopus, Penicillium* and *Monilinia* spp. (Chalutz & Droby, 1998).

Various species of soil bacteria described as antagonists of phytopathogenic bacteria and fungi produce secondary metabolites with strong antibiotic activity. One of these is pyrrolnitrin (3-chloro-4-[2'-nitro-3'-chlorophenyl] pyrrole) (Prn). The Prn-producing strains

and the purified antibiotic are effective *in vitro* as antagonists of many fungi, bacteria and yeast, including those manifested during postharvest storage (Janisiewicz, 1998).

A wide host range bacterial antagonists of a number of plant pathogens, *Pantoae* (previously *Enterobacter*) *agglomerans* strain IC1270, efficient against several soil-borne and aerial fungal diseases *in vitro* and under greenhouse conditions, produces Prn (Chernin et al., 1996). Additionally the bacterium produces a set of secreted chitinolytic enzymes consisting of two N-acetyl-β-D-glucosaminidases and a 58-kDa endochitinase (Chernin et al., 1995). In the present study we demonstrate the effectiveness of this strain against postharvest infections by *M. fructicola* and *R. stolonifer* on peaches and *P. expansum* on apples. Although chitinases have known importance for antifungal activity of many bacterial BCAs (Chernin and Chet, 2002) the predominant role of Prn in the strain IC1270 biocontrol action in postharvest was exposed by i) direct effect of the purified antibiotics on the fungi growth *in vitro* and in the wound zone, and by ii) demonstration of the fact that a mutant isolated from strain IC1270 by Tn5 insertion mutagenesis which is deficient in chitinolytic but not the antibiotic activity was equally proficient in biocontrol of *M. fructicola* and *R. stolonifer* in postharvest.

Materials and methods

Chitinolytic activity assay

Cells were seeded on plates with semi-minimal medium supplemented with colloidal chitin (0.2% w/v) and solidified with 1.5% agar as described (Chernin et al., 1995). The plates were incubated at 28°C for 96 hrs until zones of clearing of the chitin could be seen around the colonies.

Isolation of Prn

Bacteria were incubated for 4 days on minimal agar medium with glycerol 2% (v/v) as the sole carbon source. The antibiotic was extracted from agar with chloroform. The organic solution was filtered through Miracloth, washed with 0.1 M K_2HPO_4 and evaporated. The crude extracts (50 µl) were applied to HPLC system using the reverse- phase C18 column. Prn adsorption was monitored at 225 nm. HPLC-purified Prn preparations were dissolved in methanol and screened for antibiotic activity towards the tested fungi in bioassay on PDA plates.

Tn5 mutagenesis

A spontaneous mutant of strain IC1270, resistant to rifampicin (Rif^r) was used as a recipient in mating on membrane filters with *E. coli* S17-1 carrying the pUT-miniTn5-Km2 plasmid performed generally by a procedure described (de Lorenco & Timmis, 1994). The miniTn5 mutants were selected on LA plates containing Rif and kanamycin (Km) in final concentrations 40 and 50 µg/ml, respectively.

Suppression of fungal pathogens on peaches and apples in postharvest

Peaches (*Prunus persica* (L.) Batsch.) cv. "Swelling" and apples (Pirus Malus L.) cv. "Golden Delicious" were surface disinfected and uniformly wounded using a sterile needle. A drop (20 µl) of the bacteria suspension in tap water (10^7 up to 10^9 /ml) or purified Prn preparations (1 µg in 10 µl of methanol per wound) was pipetted onto the wound side. In control tap water was used instead of bacteria cells and 10 µl of methanol was applied to the wound instead of the antibiotic. The pathogen inoculums in tap water were prepared from 4- to 10-day-old cultures grown on PDA plates to yield a final concentration of 10^3 up to 10^5 CFU/ml for each pathogen. 20 µl aliquots of the pathogen suspensions were applied to the wound 2 hrs after bacteria. Fruits were air-dried, then put into firmly closed plastic trays at room temperature and about 95% relative humidity. The infection incidence and lesion diameter were observed

after 3 days and 7 days in case of *Monilina* or *Rhizopus* infection and *Penicillium* infections, respectively.

Results and discussion

***Strain IC1270 and Prn produced by this bacterium are able to suppress post-harvest fungi* in vitro**

Strain IC1270 suppresses the growth of several fungal pathogens which cause diseases in fruits post-harvest. The inhibition zones between pathogenic fungi and the strain tested were up to 14 mm for *P. expansum* and *M. fructicola* and up to 7 to 9 mm in the case of *R. stolonifer* and *B. cinerea*. Prn (0.5 µg/ml) purified from strain IC1270 was able to suppress on plates the same set of fungal post-harvest pathogens as the bacterium itself. The antibiotic drastically inhibited growth of all four tested fungi. Percentage inhibition of fungal radial growth on a plate with antibiotic as compared to a control plate was up to 70% for *P. expansum*, up to 100% for *M. fructicola* and *R. stolonifer* and up to 90% in the case of *B. cinerea*

Control of postharvest diseases on peaches and apples

Application of strain IC1270 (10^9 cfu/ml) on peaches infected by *M. fructicola* (10^3, 10^4 or 10^5 conidia/ml) reduced the incidence of the disease to 4, 46 and 80% from 60, 94 and 100% in the diseased control, respectively. In addition the size of lesions were decreased respectively from 11, 24 and 34 mm in the disease control to 5, 11 and 22 mm in peaches treated by the bacterium. Strain IC1270 (10^9 cfu/ml) also reduced the incidence of rot caused by *R. stolonifer* (10^3, 10^4 or 10^5 spores/ml) on peaches to 8, 17 and 58% from 53, 92% and 100% in inoculated control, respectively.

Tn5-mutants of IC1270 deficient in chitinolytic activity but producing Prn showed a similar efficiency towards both these pathogens as the parental strain. Moreover, Prn purified from strain IC1270 and co-inoculated with the fungi into the fruit wound completely suppressed *Rhizopus* rot and reduced the *Monilinia* rot by 83%. (Figure 1).

In addition strain IC1270 was shown to be highly efficient in control of blue mold disease caused by *P. expansum* (10^5 spores/ml) on apples: at 10^8-10^9 cfu/ml the antagonist reduced blue mold incidence by approx 95%.

The ability to produce chitinases is considered crucial for antifungal activity of strains of *E. agglomerans* biocontrol against several soil-borne fungal pathogens (Chernin et al., 1995). Contrary to these observations, the data of the present study show the importance of Prn as key factor of strain IC1270 biocontrol activity in postharvest storage. The low mammalian toxicity of Prn makes the antibiotic-producing strains reliable for some agricultural uses (Janisiewicz, 1998). The obtained results extend the range of strain IC1270 antagonistic activity indicating that the bacterium is also a perspective candidate for biocontrol of postharvest diseases of stone and pome fruits.

Acknowledgements

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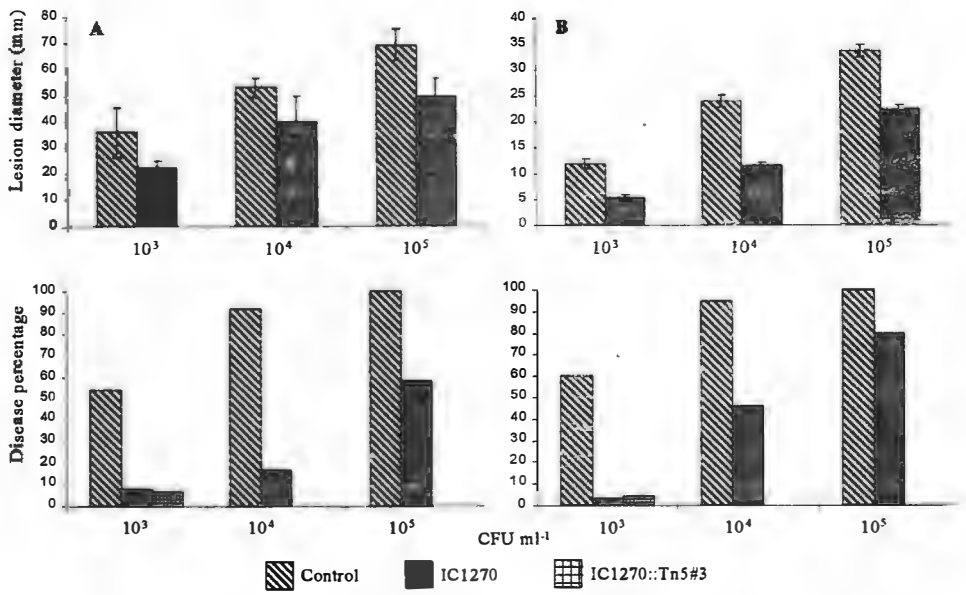

Figure 1. Dependence of the strain IC1270 biocontrol activity on concentration of *R. stolonifer* (A) and *M. fructicola* (B) used for the fruits inoculation

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Studies on the control of Chestnut Blight (*Cryphonectria parasitica* (Murr.) Barr.) by hypovirulent strains in Turkey

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Abstract: Chestnut blight is an important disease in Turkey. A survey of the chestnut growing areas of Turkey was conducted and 324 *Cryphonectria parasitica* isolates were obtained from bark samples. The isolates fall into two vegetative compatibility (EU-1 and EU-12) types. Among 324 isolates, 14 had a white phenotype and they were able to convert all the isolates of the EU-1 v-c type. The presence of dsRNA was confirmed for 7 of the 14 isolates. The performance of 7 hypovirulent isolates on young chestnut plants was evaluated during the 29 months in biocontrol study. The hypovirulent isolates caused only small and healing cankers. Similarly double inoculations with virulent and hypovirulent strains also caused small and healing cankers. It is concluded that control of the chestnut blight with hypovirulent strains will be feasible in Turkey.

Keywords: *Cryphonectria parasitica*, vegetative compatibility, conversion, hypovirulence, dsRNA

Introduction

European chestnut (*Castanea sativa* M.) is a native species of Turkey that grows in the coastal Black Sea, Marmara and Aegean Regions. Latest statistics refers the presence of 2.470.000 chestnut trees with an annual production of 61.000 tons of chestnut. Aegean Region leads with its 1.379.229 trees with total annual production of 37.230 tons (Anonymous, 1999). Chestnut blight (*Cryphonectria parasitica* (Murr.) Barr.) was first reported in Marmara Region in 1967 (Akdoğan & Erkan, 1968) and it is present now in all growing areas, threatening the cultivation of chestnut. The objectives of this study were: to search for hypovirulent *C. parasitica* strains in Turkish chestnut stands; to determine the compatibility types of *C. parsitica* isolates, to determine the conversion ability of the hypovirulent strains and to evaluate the possibility to control chestnut blight with hypovirulent strains.

Material and methods

Bark samples (20-40 pieces in each location) to obtained *C. parasitica* isolates were collected from infected chestnut groves in Aegean and Black Sea Regions from 1994 to 1998. In 1994-1995 *C.parasitica* isolates obtained from infected but living trees, however in 1998 in the Marmara Region (Izmit-Golcuk and Karamursel area), only from trees with healing cankers as described by Bissegger et al. (1997). The cultural phenotypes as white or orange was assessed as described by Bissegger et al. (1997). dsRNA isolations were performed at Swiss Federal Institute for Forest, Snow and Landscape Research by Dr. D. Rigling and Dr. U. Heiniger. Vegetative-compatibility (v-c) tests were also assessed as described by Bissegger et al. (1997). Six European tester strains (EU-1, EU-2, EU-5, EU-10, EU-12, EU-14) were

obtained from the same Institute as above. The hypovirulent isolates were tested for their ability to convert compatible orange strains according to Bissegger et al (1997).

Biocontrol test

Three years old chestnut plants grafted with 'Kara Aşı' variety were transferred to pots of 20 l and kept in the orchard of Plant Protection Research Institute, Bornova. To test the biocontrol efficiency of the hypovirulent isolates, double inoculations were performed. First a wound was applied to the depth of the cambium to each plant 40 cm above the graft union by removing a bark plug (4 mm diameter) with the help of sterile cork borer and the virulent and the hypovirulent isolates were separately inoculated into the wounds as described by Rigling (1995). One week later the second inoculation was made. A hole was applied at the lower and upper margin of the lesions, in such away that contained healthy and infected tissue and then hypovirulent strains were inoculated. The agar disks without pathogen served as control. The inoculations were conducted in 6 replicates. The canker development was measured at an interval of one month up to 29 months (Table 2).

Results and discussion

A total of 324 *C. parasitica* isolates were obtained from bark samples collected in the Aegean, Marmara and Black Sea Regions (Table 1). Fourteen out of the 324 isolates had a white cultural phenotype and all of them originated from the İzmit Province (Table 1). Seven of these isolates were tested for the presence of dsRNA and proved as positive. This was the first record of hypovirulent isolates of *C. parasitica* in Turkey (Çeliker & Onoğur, 1998). Only two vegetative-compatibility (vc) types were identified among the 310 virulent strains (Table 1). A total of 298 (96 %) virulent isolates had the vc type EU-1 and only 12 (4 %) isolates had the vc type EU-12. The vc type EU-1 was found in all the chestnut groves, whereas EU-12 was found only in one grove in the Balikesir Province in the Aegean Region . Gurer et al. (2001) also found only 1 v-c group (EU-1) in the Marmara and Black Sea Region. All the 14 hypovirulent isolates were able to convert all the 298 isolates of the vc type EU-1, suggesting that all are also of vc types EU-1. Hypovirulent strains were obtained only from İzmit Province of Marmara Region where chestnut blight was first recorded in Turkey and has caused serious damages to forests (Akdoğan & Erkan 1968). Today healing cankers are present and the hypovirus spreads naturally in this region. As the biocontrol is more successful in *C. parsitica* populations with a low number of vc types (Heiniger & Rigling, 1994), the prospects of a sustainable biocontrol with hypovirulent strains seem to be good.

Biocontrol

Cankers caused by inoculation with a virulent isolate of *C. parasitica* (V_2) could be healed when a hypovirulent isolate was inoculated close to these cankers. Two weeks after the inoculation with the hypovirulent isolate the lesion of most of the treated cankers was smaller than the lesion of the non-treated virulent cankers. Plants inoculated with the hypovirulent isolates alone showed small lesions. Within two months after the inoculation, lesion expansion of the treated cankers ceased due to formation of callus, similarly as after inoculation with only a hypovirulent isolate (Table 2). Plants inoculated with a virulent isolate showed die back above the inoculation point that progressed towards the bottom. When the study was evaluated from $17^{th}$to 29^{th} months, 3 of the 6 plants inoculated with only the virulent isolate were dead. On the other 3 plants canker development had stopped about 9-28cm above the graft union and clear callus tissue formed between infected and healthy tissue. But hypovirulent strain was not isolated from infected tissue.

Table 1. Distribution of the *C. parasitica* isolates collected from different Regions, number of white and orange isolates, vegetative compatibility types

Region	Locations and site Number	Number of isolates	Number of white isolates	Number of orange isolates EU 1	EU 12
Aegean	Izmir-Bergama	8		8	-
	Izmir-Beydag	25		25	-
	Manisa-Haciisalar,	19		19	-
	Kutahya-Simav,	6		6	-
	Balikesir- Ivrindi,	139		127	12
	Balikesir –Erdek,	22		22	-
	Canakkale-Cavuşkoy	4		4	-
Marmara	Izmit-Golcuk	17	5	12	-
	İzmit-Karamursel	45	9	36	-
	Sakarya	13		13	-
	Istanbul	19		19	-
	Bursa	2		2	-
Black - Sea	Bolu	2		2	
	Kastamonu	2		2	
	Zonguldak	1		1	
TOTAL		324	14	298	12

Table 2. Control of the canker development (cm^2) on the plants inoculated with V_2 virulent strain by the hypovirulent strains H_1-H_7 (23.7.1999- 6.12.2001)

STRAINS	04.8.1999	22.9.1999	8.12.2000	6.12.2001*
V_2	12.4	80.5	156.1	156.1 a
V_2+H_1	5.3	8,5	5.7	5.7 b
H_1	3.0	3.3	0.4	0.2 b
V_2+H_2	10.3	15.4	8.4	7.1 b
H_2	5.4	14.6	2.5	2.4 b
V_2+H_3	6.5	6.7	3.4	2.8 b
H_3	2.8	0.8	0.1	0.06b
V_2+H_4	5.3	4.5	2.2	2 b
H_4	3.2	1.6	0.5	0.3 b
V_2+H_5	7.9	9.1	6.3	3.9b
H_5	3.1	3.3	0.8	0.6 b
V_2+H_6	7.0	10.0	4.5	4 b
H_6	2.5	2.4	0.2	0.2 b
V_2+H_7	6.3	7.9	4.7	2.3 b
H_7	2.8	1.3	0.4	0.2 b

* Measurements of lesion areas are means of 6 replications. Means within a row (6.12.2001) followed by the same letter are not significantly different (P=0.01)

V_2: Virulent strain H_n: Hypovirulent strains

V_2+H_n :Virulent+Hypovirulent strains

Epicormic shoots were produced below the inoculation point. The lesions on the plants inoculated with only hypovirulent isolates had completely healed and callus tissue was formed. In the case of inoculation with both virulent and hypovirulent isolates, the lesions also healed due to similar tissue reactions and the plants maintained their normal growth and development. All hypovirulent isolates seem to be suitable for biocontrol as they all prevented mortality and induced callus growth. Statistical analysis also showed significantly reduced growth for all inoculations with hypovirulent isolates (Table 2). Finally the maintenance of the normal growth of the plants inoculated with only hypovirulent and combination of hypovirulent +virulent strains, presence of only one v-c group (EU1) in most of the chestnut groves, and presence of hypovirulent strains which were able to convert isolates of the EU-1 vc type showed that biocontrol of the chestnut blight with hypovirulent strains will be feasible in Turkey.

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Biocontrol of *Penicillium* decays with epiphytic yeasts on Satsuma mandarins

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Abstract: Evaluation of biocontrol of *Penicillium digitatum* and *P. italicum,* causing green and blue mold respectively, with epiphytic yeasts was carried out on Satsuma mandarin. Fruit samples collected from different mandarin orchards in the Aegean and Mediterranean Regions of Turkey were used for isolation of yeasts isolate. In total, 264 different isolates were obtained. The antagonistic activity of 180 of the isolates against *Penicillium* decays was tested on wounded Satsuma mandarins. From these tests, yeasts isolates exhibiting medium to high level of efficacy in reduction fruit decays (activity > 50%) were selected for further tests. The tests showed that yeast antagonists did not inhibit the pathogens through antibiotic production. TBZ and benomyl did not inhibit the growth of antagonistic yeasts, but imazalil affected their growth in culture. Three of the tested isolates (M 1/1, M 1/2 and M 1/3) colonized the wound sites, grew rapidly, and maintained their growth in temperatures of 5±1°C and 24±1°C.

Keywords: Satsuma mandarin, *Penicillium spp.* biological control, yeasts

Introduction

Satsuma mandarin (*Citrus unshiu* Marc.), most commonly grown in Izmir province, is very important export crop for Ege Region. Green and blue mold, caused by *Penicillium digitatum* Sacch. and *P. italicum* Wehmer and sour rot, caused by *Geotrichum candidum* Link, are the main diseases on Satsuma mandarins (Mahmood, 1971; Snowdon, 1990). Postharvest *Penicillium* decays induce substantial crop losses during the storage, transport and even the marketing of the Satsuma mandarin. Because of short storage period, using fungicides for control of postharvest decay increases the risk of contamination of the fruits with fungicide residues. Moreover, the development of *Penicillium* strains resistant to classic fungicides precludes their use (Eckert, 1990). Therefore, for human and environmental safety, seeking for new approaches for *Penicillium* management is unavoidable. In recent years, yeast and yeast-like organisms have been studied extensively for biocontrol of postharvest diseases of citrus and other fruits (Chalutz & Wilson, 1990; Droby et al., 1993; Kinay et al, 2001). Some yeast isolates such as *Candida oleiphila* (ASPIRE) are commercially available for control of postharvest rots of citrus fruits (Droby et al, 2001). In this study, the screening of epiphytic yeast and their antagonistic activity against *Penicillium* decays on Satsuma mandarins were examined.

Material and methods

Isolation of Epiphytic Yeasts

Potential antagonistic yeasts were isolated from surface of Satsuma mandarin in Izmir

province and Mediterranean Regions between the years of 1996 and 1998. Isolates were obtained by washing fruits individually in a flask containing 200 ml of sterile distilled water at 100 rpm for 1 hrr. One hundred µl of the washing water was then plated on nutrient yeast dextrose agar (NYDA). After 48 hrs of incubation at 25°C, yeast colonies were selected randomly and streaked on NYDA plates to obtain pure cultures (Chalutz & Wilson, 1990).

Determination of yeast biocontrol activity

Biocontrol activity of epiphytic yeasts was tested on Satsuma mandarin against *P. digitatum* and *P. italicum*. Yeast isolates were grown in NYDB for 48 hrs on rotary shaker. Cultures were centrifuged and re-suspended 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. Yeast cell suspension (30 µl) was applied into each wound, and allowed to dry for 1-2 hrs. The fruits were then inoculated with 20 µl of a pathogen spore suspension at concentration of 5×10^4 spore/ml. Fruits were kept at 20-22°C for 7 days. The percentage of infection was calculated by counting the number of decayed wounds after 7 or 10 days of incubation (Chalutz & Wilson, 1990; Wilson et al., 1993). Thirty fruits were used for a second test. Three antagonistic yeast isolates showed high efficacy on mandarin were also tested on grapefruit against *P. digitatum*.

Population dynamics of the yeast antagonist

The colonization of antagonistic yeast on the wound site of the fruit was examined. Wounds were treated with 10 µl of yeast cell suspension at concentration of 10^6 cells/ml and then cut with a sharp scalpel and homogenized by using altrotorax in 10 ml sterile water. Four fruits were used for each time. After serial dilutions, homogenate was plated on PDA containing 250 mg/l of chloramphenicol. Population size of the antagonist was determined soon after inoculation (time 0), and also after 24, 48 and 72 hours and 1 week after inoculation. Population size was also determined after 48 hrs of incubation at temperatures of 24 ±1 and 5±1°C (Droby et al., 1990).

Effect of Fungicides on Antagonistic Yeasts

To determine the sensitivity of yeast isolates to the some postharvest fungicides, a series of tests was carried out in culture. Imazalil (Magnete, 50%), TBZ (60%) and benomyl (Benlate, 50%) were added to NYDA medium at the doses of 25 and 50 µg/ml. A drop (100d µl) of yeast suspensions was seeded on these Petri dishes. After 48 hrs, the number of yeast colonies was determined (Droby et al., 1990).

Antibiosis Tests

Some of the antagonistic yeasts were tested in culture for production of antibiotic compounds. Pathogen disks (5 mm in diameter) from 5 days old PDA culture were placed on Petri dishes seeded with 100 µl of yeast cells at concentration of 10^8 spore/ml. The creation of inhibition zone around the colony growth of pathogens disks was determined and measured after seven days of incubation (Chalutz & Wilson, 1990).

Results and discussion

Antagonistic Activity of Epiphytic Yeasts

As a result of screening tests, many different yeast strains were isolated from surface of Satsuma mandarins. Among which, 180 epiphytic yeast isolates were tested for their antagonistic activity against *Penicillium* decays on wounded Satsuma mandarins. From these isolates, it was found that 20 antagonistic yeasts inhibited disease incidence by 75-100 %. Especially white and pinky pigmented yeast isolated were effective *Penicillium* decay. Three of them were selected for further tests (Figure 1). Several reports demonstrated the efficacy of

epiphytic yeast against *Penicillium* decays on other citrus fruits (Droby et al., 1989; 1990; 1993; Chalutz & Wilson, 1990; El-Ghaouth et al., 2000). In this study, the yeasts were the promising organisms for control of postharvest decays on fruits.

The antagonistic yeast isolates tested on grapefruit against *P. digitatum* inhibited the decay development by 80-100%. These results demonstrated that the yeast activity is not specific to certain hosts (Droby et al., 1990; 1993).

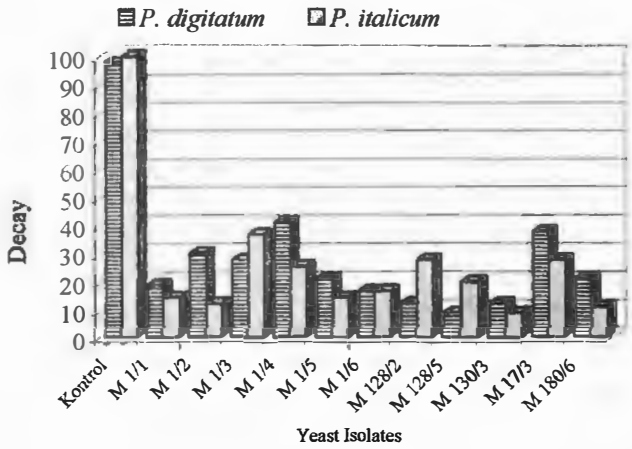

Figure 1. Effect of yeast isolates on *P. digitatum* and *P. italicum* on Satsuma mandarin

The test isolates (M1/2, M 1/3) selected from primary tests grew rapidly, colonized the wound sites and maintained in high population for seven days at 24±1°C. Isolates M1/1 and M1/2 also increased cell concentrations to 10^7 cfu/wound during 15 days storage period at 5±1°C (Figure 2). The colonization and maintenance on the wound site is very important for competition with pathogens in fruit surface (Droby et.al., 1990; Roberts, 1990).

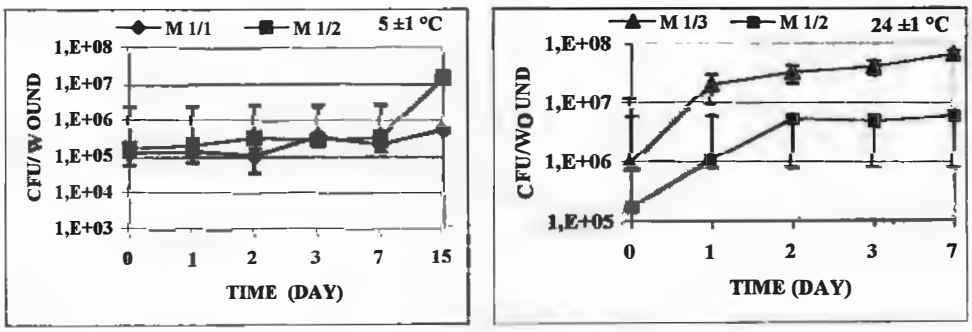

Figure 2. Population dynamic of antagonistic yeast isolates on Satsuma mandarin.
(Left: M 1/1 and M1/2 at 5 ±1 °C; right: M 1/2 and M1/3 at 24 ±1 °C)

In vitro testes revealed that TBZ and benomyl did not inhibit the growth of antagonistic yeasts but imazalil affected their growth in culture. The tests showed that yeast antagonists did not inhibit the pathogens through antibiotic production. Other mode of action of antagonistic yeast should be investigated.

Acknowledgements

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Production in solid and liquid media of chlamydospores of *Rhynchosporium alismatis*, a mycoherbicide of Alismatacae in rice crops

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Abstract: *Rhynchosporium alismatis* is a promising mycoherbicide in rice crops as this fungus has the potential to suppress several weed species and high conidial yields are easy to produce. *R alismatis* has been recently shown to produce chlamydospores on a solid medium and in a malt-extract based liquid medium (Hyphal Inclusions medium, HI). Chains of chlamydospores are observed in 2 weeks solid cultures with a production of 2.10^7 chains/cm^2. In liquid cultures, chlamydospore yields are less than 10^5 mg dry weight until 3-day growth and increase to a maximum of 1.510^6 mg dry weight at 6 day growth. The germination rate of chlamydospores harvested between 5 and 10-day growth is 80±10%. Among 10 complex liquid media tested, only the HI medium supports chlamydospore production. The culture conditions required to produce and optimize an inoculum based on chlamydospores are currently investigated.

Key words: *Rhynchosporium alismatis*, mycoherbicide, chlamydospore, fermentation, rice

Introduction

Several species in the family Alismatacae are considered as weeds in rice crops throughout the world. In Australia, the application of high doses of a bensulfuron herbicide (Londax) to control Alisma spp. is likely to have contributed to the development of weeds resistant to Londax (Graham et al., 1996). The application of a mycoherbicide as an alternative approach to the use of chemicals is currently investigated in Southern Australia. The hyphomycete *Rhynchosporium alismatis* (Oudem) J.J. Davis is a natural pathogen of several species of aquatic weeds of rice crops in New South Wales (Cother & Gilbert, 1993). Spray applications of a conidial suspension in rice fields and in the glasshouse resulted in significant suppression of weed growth (Cother & Gilbert, 1994), indicating that *R. alismatis* has potential for development as a mycoherbicide. A critical requirement for the successful application of mycoherbicides is the ability to produce desiccation tolerant fungal biomass. Recently, thick-walled spherical structures considered to be chlamydospores have been described for the first time. These chlamydospores are pathogenic to excised leaf discs of two Alismatacae weeds (Lanoiselet et al., 2001). The objective of this work was to evaluate the potential of *R. alismatis* to produce chlamydospores in solid and liquid culture for further development.

Material and methods

Isolate

R. alismatis RH 145 DAR 73154 was isolated from *Damasonium minus* (Jahromi, 2000). Stock cultures were maintained in a mixture of soil and sand (50/50%).

Inoculum production

Sub-cultures on Potato Dextrose Agar (PDA) were sampled from the soil and sand mixture and renewed every 6 months. From these sub-cultures conidia were inoculated on PDA plates and incubated at 25°C. Four day plates were washed with distilled water and the conidia suspension added to 100 ml liquid medium (10^5conidia/ml) in 250 ml flasks or sprayed evenly on the surface of 9 cm plates.

Growth and harvesting conditions

For chlamydospore production in liquid culture, a Hyphal Inclusion (HI) medium based on Czapex-Dox composition was used (g/l: K_2HPO_4: 1; sodium nitrate: 3; $MgSO_4.7H_20$: 1; KCl: 0.5; $FeSO_4.7H_2O$ 0.018; Malt extract Difco: 2.2; distilled water: 1000ml). Flasks (triplicates) were incubated at 100 rpm and 25°C for 7 days. *R. alismatis* was grown in ten complex sources (10 g/l). Yeast Extract Broth and Mycological Peptone were supplemented with glucose 2.2 g/l to provide carbon equivalent to the carbon concentration in HI medium. Cultures were vacuum-filtered on 110 mm cellulose filters, rinsed with 50 ml distilled water and allowed to dry on the bench top until constant weight. Dry mats were weighed and re-suspended in 25 ml distilled water. The suspension was blended in a commercial blender and spore counts were performed using a hemacytometer.

For chlamydospore production in solid media, potato dextrose broth and HI medium were supplemented with bacteriological agar 15 g/l (Difco). Plates were incubated at 25°C for 2 weeks. A 1 cm^2 disc of agar was taken from each plate using a stainless-steel cork borer. The disc was placed in a 7 ml tissue grinder (Kontes®) with 5 ml of distilled water and ground to separate the chlamydospores.

Chlamydospore germination

One ml of HI cultures was sampled from the third flask during growth, centrifuged at 1000 rpm and re-suspended in water. Drops of the suspension were placed on four 2 cm^2 pieces of cellophane film at the surface of water agar plates. Germination of chlamydospores was performed after 12 hrs. at 25°C. A drop of lactophenol cotton blue was added to the cellophane pieces. Germination was determined at x200 magnification on 100 chlamydospores.

Results

Chains of chlamydospores were observed on solid cultures (Figure 1) with yields at 2 weeks similar in PDA and HI agar ($2.1x10^7$±0.46 and $1.2x10^7$±0.3 chlamydospores/cm^2, respectively). Conidial production was $1.6x10^8$ ± 0.17 and $3.5x10^8$ ± 0.55 conidia/cm^2 in PDA and HI agar, respectively. In liquid culture, a thick brown ring formed at the extremities of the circular motion of the agitated broth. This ring contained mycelium and clusters of spherical structures with thickened walls, called chlamydospores. A few pellets in the culture also contained chlamydospores. After blending the whole culture, chlamydospores appeared to be single, double or formed in chains. *R. alismatis* produced high numbers of conidia and relatively few chlamydospores (Figure 2). Maximal production of conidia at 6 days was $2.2x10^7$±0.1 conidia/mg dry weight and maximal production of chlamydospores at that time was $1.5x10^6$ chlamydospores/mg dry weight. Germination of chlamydospores immediately after harvesting was determined between 4 and 9 days. Eighty percent (±10%) of the chlamydospores germinated with no significant effect of the culture age (data not shown). *R. alismatis* was grown in various complex media and conidia and chlamydospore production determined (Table 1). While all media except corn meal broth supported conidial production, only media supplemented with malt extract and nitrate (HI media) supported chlamydospore production.

Fig. 2. Growth of *Rhynchosporium alismatis* in a liquid malt-based medium (HI medium)
Croissance de *R. alismatis* dans un milieu liquide x base de malt (HI)

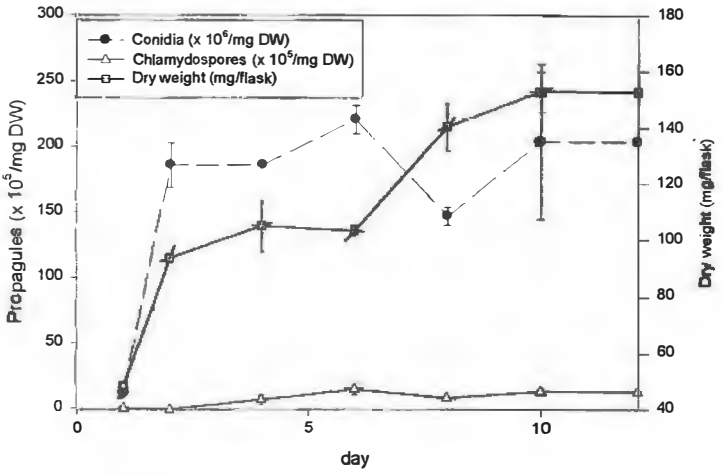

Table 1. 7-day growth of *Rhynchosporium alismatis* in complex media/Croissance at 7 days of *Rhynchosporium alismatis* in the complex media

Medium	Dry weight (mg/flask)	Conidia (x10⁶/mgDW)	Total chlamydospores (x10⁵/mg DW)[b]	pH T_0	pH 7 days
Corn Meal Broth	36.03 E[a]	0.0 C	0	8.60	6.82
Oatmeal	81.6 DE	0.2 C	0	6.71	6.77
Molasses	142.6 CD	6.6 C	0	7.00	7.27
HI Malt Difco	103.2 DE	10.6 C	10.0 A	7.37	7.57
HI Malt AmylMedia	95.2 DE	20.4 C	6.5 B	6.95	7.42
Yeast ExtractBroth	226.9 BC	86. 7 B	0	6.58	8.88
V8	309.4 AB	94.2 B	0	7.33	8.09
Pharmamedia	341.5 A	10.1.5 B	0	6.78	8.85
Lima Bean Broth	105.9 DE	106.7 B	0	6.51	8.29
Mycological Peptone	320.6 AB	179.6 A	0	6.84	8.81

[a]Numbers followed by the same letters are not significantly different (LSD test, P = 0.05)
[b]Total chlamydospores = single, double and chains

Discussion

In this study, chlamydospores were produced in potato dextrose agar and HI medium, whereas other complex sources produced conidia only. These results suggest that potato dextrose agar and HI medium contain specific nutrients inducing chlamydospore formation, as previously shown (Ciotola et al, 2000). In HI medium, carbon and nitrogen contents are relatively low (0.92 g C/l and 0.5 g N/l). The possible depletion of nitrogen and/or carbon sources may influence chlamydospore formation (Jackson & Bothast, 1990).

In our study, chlamydospores were formed in liquid and solid cultures, indicating that solid state is not required for chlamydospore formation. Nevertheless, agar concentration may increase yields as shown for the nematode-trapping *Duddingtonia flagrans* (Gardner et al., 2000). In the liquid HI medium, chlamydospores were formed, suggesting a possible development of the mycoherbicide in liquid fermentation. The ability to produce fungal inoculum in liquid culture in an advantage compared to solid state fermentation (Jackson, 1997). A method for optimizing chlamydospore production and reduce the time of incubation has to be developed. The determination of nutrient composition that induces chlamydospore formation is currently investigated.

Acknowledgments

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Influence of environmental factors on the performance of two biocontrol agents against the grey mould pathogen (*Botrytis cinerea*) in glasshouse-grown tomato crops

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Abstract: The effect of three environmental parameters (air temperature, air humidity and solar radiation) on the biocontrol effectiveness of two *Bacillus* antagonists of *Botrytis cinerea* as well as on their nutrient supplement (¼ strength TSB) was assessed during a glasshouse trial. Two main patterns of disease suppression were evident. The first was observed by the two BCAs and was dependent on the environmental factors. High temperatures (20-25°C) and low relative humidities (70-80%) resulted in higher biocontrol performance by the two BCAs. The second pattern of disease suppression was displayed by the ¼TSB, the efficacy of which was independent of the environment. There was no positive correlation between the monitored environmental factors and disease reduction by ¼TSB. The behaviour of ¼TSB may be attributable to a different mode of action.

Key words: *Bacillus*, biological control, biological control agents (BCAs), *Botrytis cinerea*, environmental parameters, tomato, tryptone soy broth (TSB)

Introduction

Many of the cultural practices in greenhouse production of tomatoes can promote disease development, particularly in situations of intensive production, where plants are often subjected to various stresses that may leave them more susceptible to disease (Nicot & Baille, 1996). The most significant factors are those related to the microclimate of the greenhouse. The range of temperature that is favourable for the development of *Botrytis cinerea*, the causal agent of gray mold, largely overlaps the range desired by growers for optimal production (Elad & Shtienberg, 1995). Condensation may occur on the plants, fostering the development of disease, due to the high relative humidity that is frequent in the greenhouse environment (Nicot & Baille, 1996). Generally, environmental factors play an important role in disease progress as well as in the functioning of biocontrol mechanisms responsible for disease suppression by the biocontrol agents (BCAs). Insufficient research efforts have been directed towards selection of BCAs with respect to the environmental conditions suitable to enhance their survival and activity *per se*. This study tested the effect of environmental parameters on biocontrol efficacy against *B. cinerea*. Two previously selected *Bacillus* isolates as well as the medium they were cultivated on, a quarter strength Tryptone Soya Broth, that previously gave effective control of *B. cinerea* in a tomato glasshouse trial were used (Tsomlexoglou et al., 2001).

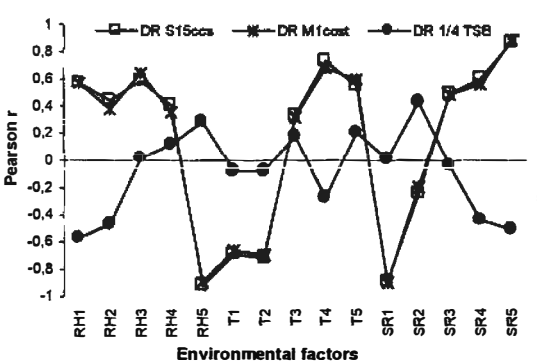
Material and methods

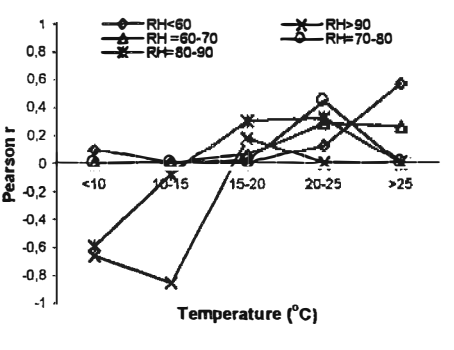
The experimental design of the glasshouse trial was a randomized block, with four replicates

and five treatments, using a total number of 192 tomato plants. The following 4 treatments were applied: the two selected *Bacillus* isolates (*B. amyloliquefaciens*- code name: S15ccs and *B. licheniformis*: M1ccst), a water control and a control sprayed with fresh ¼TSB (Tsomlexoglou et al., 2001). Disease reduction by the two BCAs and ¼TSB was studied in relation to the following recorded a-biotic factors: air temperature (T, in °C), relative humidity (RH, in %) and solar radiation (SR, in W/m^2). Sensors to monitor the above parameters were used at a height of 2 m between the blocks and data recorded hourly (CE23X data logger, Campbell Scientific). Pearson's r correlation was calculated to study the linear correlation of each factor separately with percentage disease progress. Since the continuous measurements of environmental data were not matched by equivalent observations on disease progress (weekly), "Frequency Correlation" analysis was used, in which the environmental data were summarized at the same time-scale of disease measurements without any use of the interpolated data. The frequency of an environmental factor (e.g. temperature) in different classes (e.g. 15-20°C) was set up for every time disease progressed and this was assessed once every week giving rise to time series of varying frequency in each class. In addition to analyzing the environmental data separately, a combination step of 5°C temperature at different levels of RH was also used and these frequencies were correlated (Pearson r) with the percentage disease reduction by the BCAs and the ¼TSB. Statistical analyses were done with Microsoft Excel 97 and Minitab (Release 13).

Results

The results of the correlation analyses of the recorded environmental factors with the percentage disease reduction by the two BCAs and ¼TSB are presented (Figure 1). It is clear that both *Bacillus* isolates showed almost the same correlation when environmental factors were analyzed individually (Figure 1) or in combination (Figs. 2-3). The effect of both isolates increased with positive correlation with temperatures ranging from 15 to >25°C, solar radiation from 100-400 W/m^2 and RH from 60-70%. There was a negative effect on disease reduction by the two isolates at temperatures below 15 °C, RH >90% and SR of 10-100W/m^2.





(DR: disease reduction, S15ccs=*B.amyloliquefaciens*, M1ccst=*B. licheniformis*, RH= relative humidity, RH1<60%, RH2=60-70, RH3=70-80, RH4=80-90, RH5>90, T= air temperature, T1<10°C, T2=10-15, T3=15-20, T4=20-25, T5>25, SR= solar radiation, SR1<10 Wm^{-2}, SR2=10-100, SR3=100-250, SR4=250-400, SR5>400)

Figure 1. Correlation analysis of various environmental factors with disease progress in control and percentage disease reduction by the two BCAs and ¼TSB, T in °C, RH in %, SR in W/m^2

Figure 2. Correlation analysis of disease reduction by *B. amyloliquefaciens* (S15ccs) with combined frequency classes (by 5 °C) of temperature and relative humidity (%)

For the combination of temperature and humidity (Figures 2 and 3), disease reduction by the two BCAs was negatively correlated with temperatures below 15°C at RH >90%. Thus, it was evident that the two biocontrol agents had better biocontrol performance when temperatures were above 15°C and RH below 90%. Low temperature (<15°C) and high humidity (>90%) seemed to have a negative effect on the performance of the BCAs.

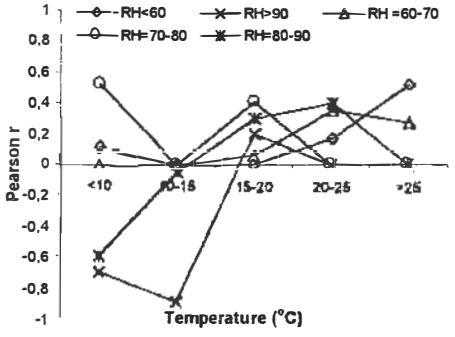


Figure 3. Correlation analysis of disease reduction by *B. licheniformis* (M1ccst) with combined frequency classes (by 5°C) of temperature and relative humidity (%).

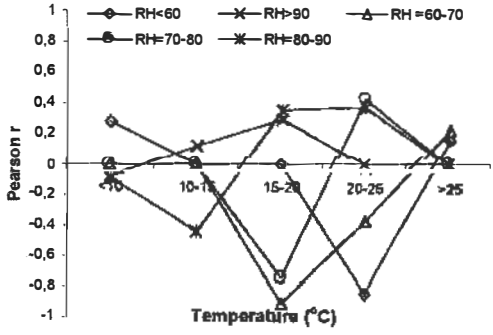


Figure 4. Correlation analysis of disease reduction by ¼TSB with combined frequency classes of temperature (by 5°C) and relative humidity (%).

For ¼TSB, none of the environmental factors individually had a strong positive correlation with the percentage disease suppression (Pearson r < 0.4) (Figure 1). High SR (> 400 W/m) as well as low RH (<60%) had a negative effect on ¼TSB performance. When analysed in combination, again temperatures between 15-25 °C at humidities ranging from <60 to 70% as well as RH between 70 and 80% at temperature 10-20°C had a negative effect on percentage disease reduction by ¼TSB (Figure 4). Some positive effect was observed at temperatures ranging from 20-25°C at RH 70-80% and 15-20°C at 80 to >90% RH.

Discussion

In this study, two BCAs and ¼ TSB that gave effective *Botrytis* control in glasshouse studies (Tsomlexoglou et al., 2001) were used. It can happen that an organism which shows outstanding antagonistic activity in preliminary screening tests may show limited or no activity *in situ* (Markellou, 1999). This is often attributed to a lowered persistence (in terms of population size and/or activity) due to different, and often varying, environmental conditions in the infection court. In this experiment, it was evident that the effectiveness of the two BCAs was responsive to environmental factors. Thus, high relative humidity (>90%), low air temperature (<15°C) and low solar radiation (<10 W/m^2) caused a decrease in the effectiveness of the two BCAs. These factors are known to promote *Botrytis* epidemics (Elad, 1989; Shtienberg et al., 1998). Temperatures between 10 and 20°C are considered optimum for spore germination, infection and sporulation but infection could also occur even at 2°C and above 25°C (Elad, 1989; Shtienberg et al., 1998). Temperatures above 15°C, humidities lower than 90% and high solar radiation (>100 W/m^2) seemed to benefit the BCAs and this might be because these are parameters that generally reduce *Botrytis* epidemics. Utilisation of climate management for disease control is increasingly regarded by tomato growers as one of the most efficient tools against *B. cinerea* (Nicot & Baille, 1996). Results have shown that in

order to maximize the performance of the particular BCAs and to limit the development of *B. cinerea* on greenhouse crops the relative humidity should be kept low. If climate management is combined with the use of the two BCAs it could result in higher disease suppression.

None of the environmental factors had a strong positive effect on disease reduction by the ¼TSB when analyzed individually (Figure 1). This effect is not easy to explain since ¼TSB had no effect on *Botrytis in vitro* or *in planta* (in controlled environments) (data not shown). The addition of ¼TSB increased the population levels of the indigenous leaf surface microflora (data not shown). Naturally occurring saprophytes are important in reducing amounts of nutrients on leaves, which would otherwise stimulate necrotrophic pathogens such as *B. cinerea* (Blakeman & Fokkema, 1982; Fokkema, 1995). Interference with the growth of saprophytes could lead to less or greater infection by pathogens (Blakeman & Fokkema, 1982; Smolka, 1993). In this study, each time there was a change in the a-biotic factors the saprophytic population could be affected in terms of organism type and activity such that there was no overall change in the influence of the a-biotic factors. Alternatively the compounds in TSB (casein and soya peptones) may affect Botrytis in a way so far not determined or induce resistance in the plants, both in a manner not influenced by a-biotic factors. Experiments to monitor populations, environmental influences and direct effects are clearly needed to determine the interactions being brought about by TSB. Future work should also determine if combinations of TSB and Bacillus BCAs could be used to enhance biocontrol of grey mould.

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Modes of action and interactions in the plant-pathogen-biocontrol agent system

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Lipid elicitors of plant defense reaction

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Abstract: Low molecular lipid substances represent specific elicitors of plant defense reaction. As for natural sterols, only ergosterol is recognized very specifically and sensitively (nM) by plant cells. Ergosterol interacts with tobacco suspension cells and trigger pH changes of extracellular medium, oxidative burst and synthesis of phytoalexins. Compared with the responses induced by cryptogein, a proteinaceous elicitor from *Phytophthora* sp., oxidative burst, pH and phytoalexin accumulation were weaker with ergosterol. Cryptogein stimulated an apparent continuous uptake of external calcium within 40 min, whereas no net uptake of external calcium occurred upon the addition of ergosterol. However, the elicitation with either cryptogein or ergosterol resulted in an increase of the fluorescence of calcium green 1 in cytosol. The use of several inhibitors of calcium channels (La^{3+}, TMB-8, verapamil, ruthenium red, nifedipine) and protein-kinase inhibitors (staurosporin, NPC-15437, H-89) suggests that the elicitation with ergosterol includes the mobilization of internal calcium stores in vacuoles mediated by IP3 and some protein kinases.

Keywords: elicitor, ergosterol, calcium channels

Introduction

The molecular mechanism of the interaction between hosts and pathogens is a subject of many studies. Usually, the products of pathogen a-virulent genes named elicitors bind to receptors and trigger a signal transduction cascade. The changes of membrane permeability leading to the influx of Ca^{2+} and H^+ and the efflux of Cl^- and K^+ followed by the oxidative burst, synthesis of phytoalexins and the activation of defense genes constitute the response of plants to microorganisms attacks. The elicitors are oligosaccharides, peptides or low-molecular substances released from hyphae or plant cell walls. Concerning lipids, it was previously reported that a specific interaction of ergosterol from *Cladosporium fulvum* with tomato cells induces extracellular pH changes. Alkanols, alkan-1,3-diols and some hydroxy fatty acids, possible products of cutin hydrolysis by fungal cutinases, were also potent H_2O_2 elicitors and enhance the effect of fungal cell wall fragments, ergosterol and chitosan (Fauth et al., 1998). Calcium channels are involved principally in signal transduction in plant cells. There were characterized in the plasma membrane, endoplasmic reticulum, tonoplast, nuclear and plastid membrane (for review see (White, 2000)).

This paper examines the mechanism of the elicitation of AOS (active oxygen species) production and H^+ transport by ergosterol, a fungal sterol, in tobacco suspension cells using several calcium channel inhibitors and protein kinases inhibitors.

Material and Methods

Plant cells harvested in exponential phase of growth were filtered, washed and re-suspended in elicitaation buffer (0.1 g FW/ml). After a 2 hrs equilibration period, the cells were treated with the elicitors and the inhibitors. The production of AOS was determined by luminol reaction in BioOrbit 1253 Luminometer. The pH changes were registered every 5 min after the addition of elicitor. Ca^{2+} uptake was determined by monitoring the variation of $^{45}Ca^{2+}$ amount associated with the cells as described by Tavernier et al. (1995). Phytoalexins were extracted from the medium 24 h after elicitor treatement and their GC analysis was performed according to Milat et al. (1991). The calcium fluxes in tobacco suspension cells were monitored using Calcium Green 1 acetoxymethylester (CG-1), and a Leica TCS 4D confocal microscope equipped with an argon-krypton laser (excitation filter 488 nm, barrier filter LP515).

Results and discussion

Influence of the sterol structure in elicitation of tobacco cells

Tobacco cells were treated with different sterols; ergosterol, 9-dehydroergosterol, ergocalciferol, cholesterol and 7-dehydrocholesterol. The production of AOS was assessed using 1.2 μM of each sterol studied. Typical results are shown in Fig. 1. The interaction of tobacco cells with sterols presents some specific features because only ergosterol and 9-dehydroergosterol were effective, 7-dehydrocholesterol was slightly efficient whereas cholesterol and ergocalciferol were inefficient. This suggests the significance of two double bonds in the ring B and that of ergosterol side chain for the specificity of binding to a possible receptor.

Ergosterol-concentration dependence of the AOS production and pH changes

The production of AOS (10 μM hydrogen peroxide) was maximal and saturated with 30 nM ergosterol. The effect on pH changes was more noticeable and even at low ergosterol concentration (such as 5 nM), a pH of 0.31 was observed.

Calcium-distribution changes in tobacco cells visualized by fluorescence confocal microscopy

The cells were incubated with the elicitors for the time necessary to obtain maximal elicitation effect, i.e., 10 and 30 min for ergosterol and cryptogein, respectively. Elicitation with either cryptogein or ergosterol resulted in an increase of the fluorescence of calcium green 1 in cytosol by comparison with cells incubated without elicitor.

Ergosterol-elicited influx of calcium into tobacco cells

Elicitation of tobacco cells by cryptogein resulted in a massive uptake of external $^{45}Ca^{2+}$ whereas no net change in calcium content occurred upon the addition of ergosterol although ergosterol triggered an increase in the cytosolic Ca^{2+} level as shown by confocal microscopy with calcium green 1. However, in the case of cryptogein/tobacco-cell interaction, this longterm accumulation of calcium has been shown to contribute to the reinforcement of cell wall (Pauly et al., 2001).

Role of calcium channels in the elicitation of tobacco cells with ergosterol

Several calcium-channel inhibitors were used to analyze the calcium fluxes induced in tobacco cells by the elicitation with ergosterol. The effects of the inhibitors are shown in Table 1. $LaCl_3$, at micromolar concentrations, inhibited both the AOS production and extracellular medium alkalization induced by cryptotein but it only partially inhibited these responses when ergosterol was used as elicitor. Micromolar La^{3+} concentrations inhibit the

activity of most channels in the plasma membrane whereas milimolar concentrations are necessary to affect vacuolar channels (Pineros & Tester, 1997). Nifedipine inhibit voltage dependent calcium channels tomato and wheat plasma membrane and tonoplast (Pineros & Tester, 1997). It inhibited neither the effect of cryptogein nor ergosterol. Ruthenium red is the inhibitor of voltage-dependent calcium-channels on plasma membrane and cADPR dependent channels on tonoplast (Muir et al., 1997). It had a pronounced effect on the elicitation with cryptogein but it affected only slightly the elicitation with ergosterol. On the other hand, TMB-8, antagonist of IP_3-mediated intracellular calcium release (Muir et al., 1997) did not significantly inhibit AOS synthesis and pH changes induced by cryptogein but it strongly reduced the extracellular alkalization and the AOS synthesis induced by ergosterol. This could suggest that the elicitation with ergosterol does not involve the participation of plasma membrane calcium channels. However, it involves the mobilization of internal calcium stores in vacuoles probably mediated by IP3 and not by cADPR as in the case of abscissic acid (White, 2000). Verapamil, inhibitor of plasma membrane and tonoplast voltage-dependent calcium-channels and IP_3 dependent calcium channels on tonoplast (Pineros & Tester, 1997), inhibited pH changes induced by ergosterol but not those induced by cryptogein.

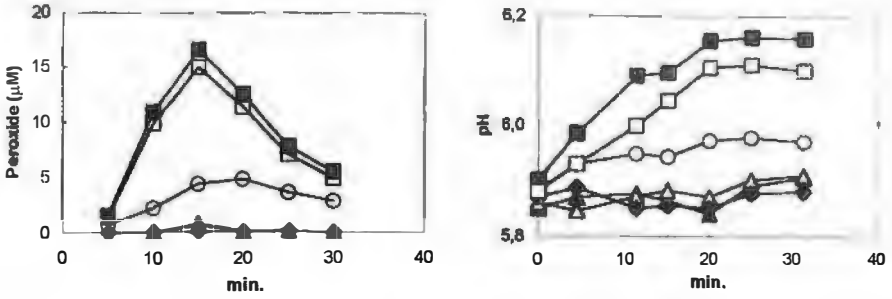

Figure 1. Comparison of eliciting activities of different sterols; A, AOS; B, pH changes; 1.2 µM sterols; ■,ergosterol; □, dehydroergosterol, ▲, ergocalciferol; ×, cholesterol; ❍, dehydrocholesterol; ♦, 50 µL of ethanol as blank

Role of protein kinases in the elicitation of tobacco cells with ergosterol

The inhibitor of phospholipid/Ca-dependent protein kinases staurosporin inhibits either the effect of cryptogein or the effect of ergosterol. The selective inhibitor of protein kinase C NPC-15437 inhibits the effect of ergosterol but does not inhibit the effect of cryptogein. The selective inhibitor of protein kinase A, H-89l, inhibited neither the effect of cryptogein nor that of ergosterol. The results clearly show that protein kinase C and not protein kinase A participates in the transduction of the signal elicited by ergosterol.

Ergosterol-induced synthesis of phytoalexins

Addition of ergosterol to tobacco suspension cells induced the synthesis of capsidiol. The concentration of capsidiol induced by ethanol as a blank was 26.9 µg/g of cell dry weight, whereas that induced by 25 nM ergosterol was 302 µg/g. The synthesis of capsidiol increased when the concentration of ergosterol was increased up to 2.5 µM in spite of the fact that the AOS synthesis and pH changes were saturated at 25 nM ergosterol.

Table 1. Effect of calcium-channel inhibitors. The results (± SE) represent the mean activity obtained after 15 min of elicitation compared with the non-inhibited reaction

Inhibitor	25 nM cryptogein		200 nM ergosterol	
	AOS (%)	ΔpH (%)	AOS (%)	ΔpH (%)
100 µM $LaCl_3$	32 (11)	31 (2)	100 (10)	75 (10)
300 µM $LaCl_3$	6 (1)	1.2 (3)	54 (4)	61 (8)
100 µM r.red	-	47 (18)	-	87 (6)
200 µM r.red	-	22 (7)	-	80 (12)
100 µM verapamil	67 (26)	90 (9)	27 (6)	55 (16)
200 µM verapamil	56 (21)	91 (14)	30 (8)	48 (20)
50 µM TMB-8	96 (2)	92 (4)	33 (7)	32 (2)
100 µM TMB-8	97 (4)	97 (8)	26 (11)	24 (4)
100 µM nifedipine	-	105 (13)	-	95 (2)
200 µM nifedipine	-	99 (1)	-	91 (1)

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Induced resistance by *Pseudomonas aeruginosa* 7NSK2: bacterial determinants and reactions in the plant

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Abstract: The rhizobacterium *Pseudomonas aeruginosa* 7NSK2 produces secondary metabolites such as pyochelin (Pch), its precursor salicylic acid (SA) and the phenazine compound pyocyanin (Pyo). Both 7NSK2 and mutant KMPCH (Pch^-, SA^+) induced resistance to *Botrytis cinerea* in wild type tomato but not in transgenic *NahG* plants, in which SA is converted to catechol. In addition, SA^- mutants of both strains, loose the capacity to induce resistance. These findings suggest a role for SA-dependent plant defence. On tomato roots, however, KMPCH but not 7NSK2 produced SA although 7NSK2 produces SA *in vitro*. We hypothesize that *in vivo*, SA is efficiently converted to Pch. We have shown in *in vitro* experiments that Pch production by 7NSK2 increases in the presence of L-cysteine. Pch alone however, appeared not be sufficient to induce resistance. To study a role for Pyo in induced resistance by 7NSK2, a Pyo^- mutant of 7NSK2, PHZ1, was generated, which is mutated in the *phzM* gene encoding an O-methyltransferase. PHZ1 was unable to induce resistance to *B. cinerea* while complementation for Pyo production restored induced resistance. These results demonstrate that Pyo is a crucial bacterial determinant in induced resistance in wild type *P. aeruginosa*. Co-inoculation of PHZ1 with mutant 7NSK2-562 (Pch^-, SA^-, Pyo^+) restored the ability to induce resistance which points to a synergistic effect of Pyo and Pch in induced resistance. By using the cDNA-AFLP technique we could not observe changes in gene-expression in leaves of tomato plants colonized by the resistance inducers 7NSK2 and KMPCH. This indicates that induced systemic resistance by 7NSK2 and KMPCH involves priming.

Key words: phenazines, systemic acquired resistance, gene-expression, cDNA-AFLP

Introduction

Some rhizobacteria can induce systemic resistance (ISR) in various plants species. Both bacterial determinants and signaling pathways that are involved in the activation of plant defence appear to vary among bacterial strains and pathosystems. For the bacterial strain *P. aeruginosa* 7NSK2, SA has been demonstrated to be important for the induction of resistance to *B. cinerea* in bean and tomato (De Meyer et al., 1999; De Meyer & Höfte, 1997). Under iron-limitation, SA-deficient mutants were unable to induce resistance. Moreover, it was illustrated that bacterial SA produced by the SA-producing mutant KMPCH (Pch-negative) induced the SA-biosynthesis enzyme PAL in bean roots (De Meyer et al., 1999). These results clearly demonstrate that bacterial SA induces SA-dependent plant defence. In *P. aeruginosa*, SA is produced from chorismate via the shikimate biosynthesis pathway (Serino et al., 1995) and is the precursor of Pch. Since mutants in SA-biosynthesis are also Pch-negative, a role for Pch in induced resistance by the wild type (WT) strain 7NSK2 cannot be excluded.

In the present work, we demonstrate that in tomato, KMPCH induces resistance through activation of SA-dependent defence as earlier described by De Meyer et al. (1999) although no changes in gene-expression patterns were observed compared to WT plants. However, we provide evidence that for the WT strain 7NSK2 a combined action of Pch and Pyo is more likely to be involved in induced resistance. Pyo (5-methyl-1-hydroxyphenazinium) is a blue phenazine compound produced by *P. aeruginosa* and is considered to be a virulence factor in clinical isolates of *P. aeruginosa* (Britigan et al., 1997).

Material and methods

Bacterial strains

Bacterial strains derived from the WT strain 7NSK2 are represented in Table 1.

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

Strain	Metabolite	Characteristics	R
7NSK2	Pch^+, SA^+, Pyo^+	Wild type	+
7NSK2-562	Pch^-, SA^-, Pyo^+	*pchA* replacement mutant of 7NSK2	-
KMPCH	Pch^-, SA^+, Pyo^+	Chemical mutant of MPFM1	+
KMPCH-567	Pch^-, SA^-, Pyo^+	*pchA* replacement mutant of KMPCH	-
PHZ1	Pch^-, SA^-, Pyo^-	MiniTn*Pho*A3-mutant in *phzM*	-

For references see De Meyer & Höfte (1997) and De Meyer et al. (1999): relevant characteristics and ability to induce resistance (R) in tomato to *B. cinerea*. Pch: Pyochelin, SA: Salicylic acid, Pyo: Pyocyanin.

Assay for induced resistance

Bacterized tomato plants and control plants were infected with *Botrytis cinerea* R16 as described in Audenaert et al. (2002). Transgenic *NahG* tomato plants (Brading et al., 2000) were infected similarly.

Determination of SA on tomato roots and in tomato leaves

Free SA was measured as described in De Meyer et al. (1999). Samples were taken from control plants and plants colonized by bacteria, 4 weeks after seedling transfer. Each sample consisted of 2.5 g of plant material pooled from 5 individual plants.

Gene-expression analysis

To analyse changes in gene-expression upon colonization by bacterial resistance inducers, total RNA of leaves of 5 week-old plants was extracted using a phenol/SDS method. Subsequently, mRNA was isolated using the Dynabead® technology. Finally, cDNA-AFLP analysis was performed with two selective nucleotides.

Results and discussion

KMPCH and 7NSK2 induce resistance in wild type but not in NahG tomato plants

As observed in Table 2, both SA^- and Pyo^- mutants loose the ability to induce resistance to *B. cinerea* in tomato. In addition, all resistance inducing strains loose their ability to induce resistance in *NahG* plants in which SA is converted to catechol. This indicates that a functional SA-response in the tomato plants is necessary for induced resistance by both 7NSK2 and KMPCH.

Table 2. Ability of 7NSK2 and its mutants to induce resistance to *B. cinerea* in WT and *NahG* tomato

Strain	Resistance in WT	Resistance in *NahG*
7NSK2	+	-
7NSK2-562	-	-
KMPCH	+	-
KMPCH-567	-	-
PHZ1	-	-

KMPCH but not 7NSK2 produces detectable amounts of SA on tomato roots although both strains cause increased SA-levels in tomato leaves

To validate the role of SA-dependent plant defence in induced resistance by KMPCH and 7NSK2, SA-levels were measured in leaves and roots colonized by KMPCH and 7NSK2. Surprisingly, KMPCH colonization but not 7NSK2 colonization resulted in increased levels of free SA on tomato roots compared to control plants. This result suggests that 7NSK2 does not produce SA on tomato roots. However, both KMPCH and 7NSK2 induced increases in free SA in tomato leaves, which points to an activation of SA-dependent defence by 7NSK2 and KMPCH at the site of expression of resistance.

The presence of L-cysteine and SA are sufficient to increase pyochelin production

We wanted to clarify why SA is produced by 7NSK2 *in vitro* where no detectable increases in SA are observed *in vivo* and showed that the production of Pch increased in the presence of L-cysteine (data not shown). It is known that Pch is derived from SA and two molecules of L-cysteine (Reimann et al., 1998). It is not unlikely that L-cysteine, which is present in tomato root exudates (Gamliel and Katan, 1992), favours the conversion of SA to Pch leaving no detectable amounts of SA on the root surface. This implicates that for WT strain 7NSK2, SA is probably not directly involved in induction of resistance.

Pyocyanin is crucial for 7NSK2 to induce resistance to B. cinerea in tomato and acts in concert with pyochelin

Since a Pch^- mutant of 7NSK2 i.e. 7NSK2-562 no longer induced resistance to *B. cinerea* in tomato (Table 2), we verified if Pch was sufficient for induction of resistance. This was, however, not the case. We hypothesized that Pch acted synergistically with a second molecule to induce resistance. Since it was shown before that high concentrations of purified Pyo can induce resistance to *B. cinerea* in bean, we investigated if this phenazine molecule was the accomplice of Pch in induced resistance by 7NSK2. Mutant PHZ1 is not able to produce Pyo due to an insertion in *phzM, which* encodes an O-methyl transferase (Mavrodi et al., 2001). Using mutant PHZ1 we were able to demonstrate that Pyo was crucial for induced resistance by 7NSK2. In addition, complementation of PHZ1 or co-inoculation of PHZ1 and 7NSK2-562 (Pch^-, Pyo^+) restored the ability to induce resistance. This result confirms the synergistic role of Pyo and Pch in induced resistance to *B. cinerea* in tomato. A clue for the mechanistics of induced resistance by Pyo and Pch comes from knowledge on the infection process of clinical *P. aeruginosa* isolates in pulmonary lung cells. Pyo is considered to be a pathogenicity factor undergoing redox cycling which generates superoxide and H_2O_2. These moderately active reactive oxygen species (ROS) are converted to the very reactive OH-radical in the presence of Fe-Pch which causes oxidative damage to epithelial cells (Britigan et al., 1997). Since it is known that ROS are important second messengers in plant defence it is possible that induced resistance by 7NSK2 is realized through generation of ROS.

Resistance inducing strains do not cause changes at a level of gene-expression
To investigate eventual changes upon root colonization by resistance inducing strains at a level of gene-expression, we performed cDNA-AFLP on leaf material of control plants and plants colonized by KMPCH or 7NSK2. We tested 60 primer combinations with 2 selective nucleotides. Not a single difference at the level of mRNA could be detected on leaf material of 5 week-old tomato plants. This result suggests that either transcriptional changes occurred earlier in time leaving no detectable changes after 5 weeks, or that induced systemic resistance by rhizobacteria involves priming and potentiation of plant defence rather than activation of new defence compounds.

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Modes of action of *Pantoea agglomerans* CPA-2, an effective antagonist against postharvest pathogens on fruits

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Pantoea agglomerans CPA-2 is an effective antagonist against postharvest pathogens such as *Penicillium* spp. on citrus fruits and *Penicillium expansum, Botrytis cinerea* and *Rhizopus stolonifer* on apple. Its mode of action, however, is unknown. Possible mechanisms studied in this project were antibiosis, induced resistance and competition.

To study antibiotic production, we did streak and overlay assays with *P. agglomerans* CPA-2 and the pathogens *P. digitatum* and *P. italicum* on different kinds of media. The results were compared with the same assays done with *Pantoea agglomerans* A111, a known producer of herbicolin-type antibiotics. Clear inhibition zones were observed in the assays with *P. agglomerans* A111, but not with *P. agglomerans* CPA-2. We were also unable to detect antibiotic production by *P. agglomerans* CPA-2 on citrus fruits. From these results we can assume that *P. agglomerans* CPA-2 does not produce antibiotics.

The ability of *P. agglomerans* CPA-2 to induce resistance to *Penicillium* spp. on oranges was studied by measuring phenylalanine ammonia lyase and peroxidase activity in the orange peel at different time points after inoculation with the antagonist and/or the pathogen. There was no significant augmentation of enzyme activity after inoculation of oranges with *P. agglomerans* CPA-2 in the presence or absence of the pathogen. In addition, *P. agglomerans* was only effective when in close contact with the pathogens.

Competition for nutrients was studied using tissue culture plates with cylinder inserts containing a 0.45 µm membrane as described by Janisiewicz et al. (2002). This method allows to study competition for nutrients without competition for space since physical contact between pathogen and antagonist is avoided. The presence of *P. agglomerans* in the tissue culture wells clearly decreased germination of *Penicillium* spores present in the cylinder when the nutrient source was diluted orange extract or diluted potato dextrose broth. Germination of *Penicillium* spores, however, was completely inhibited when pathogen and antagonist were no longer separated by the membrane. These results indicate that competition for nutrients is one of the modes of action of *P. agglomerans* CPA-2, but that physical contact between pathogen and antagonist is important for an effective control.

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Biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* in the greenhouse by a *Serratia plymuthica* strain with multiple mechanisms of antifungal activity

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Abstract: Plant-associated, non-hemolytic strain IC14 of the Gram-negative bacterium *Serratia plymuthica* was shown to be able to suppress a wide range of phytopathogenic fungi *in vitro*. The strain produced antibiotic pyrrolnitrin and siderophores, possessed proteolytic and chitinolytic activities and secreted the plant growth hormone indolyl-3-acetic acid. An endochitinase with an apparent molecular mass of 58-kDa was estimated as the main chitinolytic enzyme. In greenhouse experiments, the strain efficiently protected cucumber against *Botrytis cinerea* and *Sclerotinia sclerotiorum* by repeated foliar application one day before and immediately prior to fungal infection, reducing the number of affected leaves by 76 and 84%, respectively. Two mutants, one with increased chitinolytic activity and the second deficient in chitinolytic activity, were obtained by Tn5 insertion mutagenesis. Neither mutant differed appreciably from the parental strain in the production of other antifungal compounds or in protection of plants against *B. cinerea* and *S. sclerotiorum*, suggesting that chitinolytic activity less essential for biocontrol of these pathogens by strain IC14.

Keywords: chitinases, pyrrolnitrin, siderophores, indolyl-3-acetic acid, grey mold, white mold

Introduction

The necrotrophic fungi *Botrytis cinerea* Pers; Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary are among the world's most dangerous fungal plant pathogens due to their effects on flowers, leaves, fruits or stems under high humidity or when free moisture is present on the plant surface. Biocontrol is an environmentally friendly and efficient alternative to chemical fungicide management of these pathogens. However, of the approx 80 commercial biocontrol agents (BCAs) available today for use against crop diseases, only a few are specifically recommended for use against these fungi (Whipps & Davies, 2000).

In the present work we describe the soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity, which is able to protect cucumber against *B. cinerea* and *S. sclerotiorum* leafs infection under greenhouse conditions. Strain IC14 was isolated from the soil around melon roots that had survived a *Fusarium oxysporum* f. sp. *melonis* infection background in an experimental field in Rehovot, Israel. The objectives of the study were: (i) to study the strain IC14 antagonistic activity against economically important plant pathogenic fungi *in vitro* and under greenhouse conditions; (ii) to determine the mechanisms of this bacterium antifungal activity; (iii) to study the role of chitinases in the biocontrol activity of strain IC14 against *B. cinerea* and *S. sclerotiorum* foliage diseases.

Material and Methods

Preparation of extracellular proteins and detection of chitinolytic enzymes

The procedure was generally performed as described previously (Chernin et al., 1995). Liquid or solid synthetic medium with 0.2% (w/v) colloidal chitin as the sole carbon source was used to induce chitinolytic activity of the bacteria. Secreted proteins denaturated with sodium dodecyl sulfate (SDS) were separated by 10% polyacrylamide gel electrophoresis (PAGE) and then reactivated by removing SDS using the casein-EDTA procedure. Enzyme activity was detected on gels by using fluorescent 4-methylumbelliferyl analogues of dimeric, trimeric and tetrameric chitin as substrates.

Antibiotic compound purification and assay

The crude extracts of the antibiotic from cells grown on PDA plates were prepared as described previously (Chernin et al., 1996). The extracts were fractionated by thin-layer chromatography (TLC) and the fraction with antibiotic activity was analyzed by high-pressure liquid chromatography. Antibiotic absorption was monitored at 225 nm. Samples were collected, concentrated and tested in a bioassay against *R. solani*.

Tn5 mutagenesis

A spontaneous mutant of strain IC14, resistant to rifampicin (40 μ g/ml) was used as a recipient in mating with *E. coli* S17-1 carrying the pUT-miniTn5-Km2 plasmid, generally following the procedure described by de Lorenzo & Timmis (1994).

Greenhouse experiments

For protection of cucumber against *B. cinerea*, cucumber (*Cucumis sativus* L. cv. "Cfir 413") seedlings were sprayed with the bacterial (10^6 cells/ml) suspension. Then a *B. cinerea* spore suspension (1 x 10^6 spores/ml) was applied to the each true leaf in triple drops. To assay for protection against *S. sclerotiorum* agar disks covered with 5 to 7-days-old mycelium were taken from PDA plates and applied to the leaves. In both cases seedlings were incubated at high humidity (RH 90-95%) and the effect of the bacterial application was monitored 3 to 4 days after infection when obvious spots of necrotic tissue were observed on infected leaves of plants used as control. Disease was estimated as percentage of necrotic zones per total amount of drops of inoculum of *B. cinarea* or of agar discs covered with *S. sclerotiorum* mycelium applied to the leaf surface.

Results and discussion

Production of chitinases by strain IC14 and its miniTn5 mutants

Strain IC14 hydrolyzed colloidal chitin after 72 to 96 hrs of growth on solid medium supplemented with colloidal chitin as the sole carbon source. Clear zones of chitin degradation around the growing bacteria were observed. Two miniTn5-insertional mutants differing from strain IC14 in their chitinolytic activity were obtained. One of them, IC14::miniTn5#9, revealed increased chitinolytic activity, while the another one (IC14::miniTn5#18) failed to hydrolyze colloidal chitin in the plate test.

Identification of chitinolytic enzymes

The extracellular proteins were renatured after SDS-PAGE and their chitinolytic activity determined with a set of three fluorescent 4-methylumbelliferone (4-MU) chitin derivatives. Only one main band corresponding to chitinase with an apparent molecular mass of 58-kDa (CHIT58) was detected in the extracellular proteins of strains IC14 and IC14::miniTn5#9, but not in IC14::miniTn5#18 growing with colloidal chitin. Additionally, a poorly visible band with an apparent molecular mass of about 100 kDa was observed with 4-MU- GlcNAc in the

extracellular proteins from strain IC14 and IC14::miniTn5#9, but not in IC14::miniTn5#18 (data not shown).

***Antagonism* in vitro**

Isolate IC14 suppressed the growth of various fungal phytopathogens *in vitro*, including the soil-borne *F. oxysporum* f. sp. *lycopersicum*, *F. oxysporum* f. sp. *melonis*, *Pythium aphanidermatum*, and *R. solani*, the foliage plant pathogens *Alternaria alternata*, *B. cinerea* and *S. sclerotiorum*, and fungi which cause diseases of fruits in post-harvest storage (*Penicillium expansum*, *Monilinia fructicola* and *Rhizopus stolonifer*). Strain IC14 and the miniTn5 mutants differed in chitinolytic activity are equally proficient in suppression of *B. cinerea* and *S. sclerotiorum* growth *in vitro*.

Production of antibiotic and other antifungal compounds

The ability of strain IC14 to produce the strong antifungal antibiotic pyrrolnitrin was detected by PCR assay with specific primers to the conserved region of gene *prnC* of *P. fluorescens*, by TLC and HPLC analyses of purified antibiotic activity, and by positive bioassay test with *R. solani*. The same levels of pyrrolnitrin production and antifungal activity were observed in TLC and HPLC analyses of crude materials isolated from both IC14::miniTn5#9 and IC14::miniTn5#18 mutants. In addition strain IC14 and both its miniTn5 mutants have proteolytic activity and produce still non-identifiede siderophore(s), however, are apparently unable to produce β -1,3-glucanases or HCN and were found non-hemolytic. The parental strain and the mutants were equally proficient in the production of indolyl-3-acetic acid (IAA) *in vitro* in the presence of L-tryptophan as precursor (data not shown).

Greenhouse experiments

The strain IC14 efficiently protected cucumber against *B. cinerea* and *S. sclerotiorum* by foliar application reducing the number of affected leaves by 76 and 84%, respectively, while neither mutant differed appreciably from the parental strain in protection of plants against *B. cinerea* and *S. sclerotiorum*, suggesting that chitinolytic activity is less essential for biocontrol of these pathogens by strain IC14 (Table 1). A large number of microorganisms, including fungi, bacteria, and actinomycetes, as well as plant species, possess the ability to excrete cell-wall hydrolases such as chitinases, β -1,3-glucanases, and proteases. These hydrolases play an important role in the reactions between BCAs and pathogens (reviewed by Chernin & Chet, 2002). The ability to produce chitinases is considered crucial for antifungal activity of strains of *E. agglomerans* (Chernin et al., 1995) and *S. plymuthica* strain HRO-C48 (Frankowski et al., 1998), both efficient BCAs of several diseases caused by soil-borne fungal pathogens. Contrary to these observations, in the case of strain IC14, the same level of antifungal activity *in vitro* and *in vivo* was observed with the parental chitinolytic strain and its two miniTn5 mutants, one of which (IC14::miniTn5#9) is a super-producer of chitinase, while the other (IC14::miniTn5#18) is deficient in chitinolytic activity. These data indicate that other antifungal compounds produced by strain IC14, which are not chitinases, play a key role in the strain's activity, at least against *B. cinerea* and *S. sclerotiorum*. Actually, strain IC14 and both its mutants did not differ in their ability toproduce pyrrolnitrin, siderophores or proteolytic enzymes, suggesting the predominant role of either one or all of these compounds in the strain IC14's biocontrol activity towards foliage fungal pathogens, while the role of chitinolytic enzymes appears to be less essential. The ability to produce the plant-growth-promoting IAA and its lack of hemolytic activity at human body temperature could be considered additional advantages for this bacterium's further development as a BCA of a wide range of crop diseases.

Table 1. Effects of the biocontrol bacteria *Serratia plymuthica* on mold sevreity (%) caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum* in cucumber seedlings

Fungus	Untreated control	*S. plymuthica* strain		
		IC14	IC14::miniTn5#9	IC14::miniTn5#18
B. cinerea	62.7 b	13.7 a	15.4 a	10.8 a
S. sclerotiorum	69.4 b	12.0 a	31.4 a	24.3 a

Data are means of four independent experiments. Homogeneity of the variances between repetitions in all experiments was proven with Bartlett test (>0.05). Different letters in the same row indicate significant differences between means using the All Pairs Tukey-Kramer test ($\alpha = 0.05$, $P < 0.001$).

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Expression of defense-related genes in cucumber treated with culture filtrate of plant growth-promoting fungus, *Penicillium simplicissimum* GP17-2

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Abstract: Hypocotyls of cucumber seedlings which their roots were treated with culture filtrates (CFs) of plant growth-promoting fungi (PGPF), induced lignification at attempted penetration points of *Colletotrichum orbiculare*. The CFs of PGPF isolates generated H_2O_2 from cucumber fruit disks. Maximum chemiluminescence activity was shown using the CF of *P. simplicissimum* GP17-2. Hypocotyls of cucumber seedlings which their roots were treated with the culture filtrate of *P. simplicissimum* GP17-2, expressed peroxidase and chitinase genes between 2 and 24 hrs after CF-treatment while expression of β-1,3-glucanase gene was not detected even after 24 hrs CF-treatment. Under challenge inoculation with *C. orbiculare*, activation of peroxidase and chitinase genes was more rapid and more prominent in the hypocotyls of CF-treated cucumber. A similar result was also found inβ-1,3-glucanase gene. These results suggest that priming effect occurred in the cucumber plants treated with CF of *P. simplicissimum* GP17-2.

Key word: plant growth-promoting fungi, induced systemic resistance, defense-related genes

Introduction

Induced systemic resistance (ISR) is an important component of disease resistance in plants. It is associated with an enhanced potency of activating various defense responses. So far, some researchers reported that plant growth-promoting rhizobacteria (PGPR) could ISR in plants against several diseases without activation of pathogenesis-related (PR) genes (Pieterse et al., 2001), while others reported that induced resistance by other strains lead to accumulation of PR-genes (Park et al., 2000). The mechanisms of PGPR-mediated ISR are now becoming more complex. As for PGPF, however, the mechanism of ISR is not well known. We have previously shown that a PGPF, *Penicillium simplicissimum* GP17-2, could induce systemic resistance activity in cucumber against anthracnose caused by *Colletotrichum orbiculare* and could effectively suppress disease development (Koike et al., 2001). The objective of this study was to determine whether PGPF strain, *P. simplicissimum* GP17-2, which ISR activity in cucumber, could activate PR-genes.

Materials and methods

Microorganisms, plants and culture conditions

Plant growth-promoting fungi, *P. simplicissimum* GP17-2, *Fusarium equiseti* GF18-3 and *Phoma* sp. GS8-2, were cultured in potato-dextrose broth without shaking at 25°C for 10 days in darkness. The fungal mats were separated from the culture filtrate (CF) by using five layers

of cheesecloth. The anthracnose pathogen, *C. orbiculare* isolate 104T, was maintained at 25°C on 3% potato dextrose agar for 7 days. Cucumber seeds (*Cucumis sativus* L. cv. Gibai) were sown in moist sterilized filter paper and incubated for 7 days in the dark at 25°C.

Induction and inoculation assay

Cucumber roots of each seedling were individually dipped in the CFs of three isolates of PGPF contained in tubes and incubated for 24 hrs. After incubation, the hypocotyls of the seedlings were inoculated in 10 locations with 5 μl *C. orbiculare* spore suspension (10^5 spores/ml).

Observation of lignin deposition

The degree of lignin deposition was evaluated in etiolated hypocotyls of cucumber seedlings induced by CFs of PGPF isolates following challenge inoculation with *C. orbiculare* 24 hrs after CF-treatment using Toluidine-blue O or Phloroglucinol-HCl stainings.

Measuring of hydrogen peroxide generation using a chemiluminescence assay

H_2O_2 generation was evaluated based on luminol-dependent chemiluminescence emitted from cucumber fruit disks. The surfaces of cucumber disks were coated with a mixture of Tris-HCl buffer (pH7.4) containing 1mM luminol and CFs of PGPF. The emission of the chemiluminescence from the cucumber disk was recorded based on percent relative strength as compared to H_2O_2 treated control.

RNA extraction and gel blotting

Cucumber hypocotyls were harvested from cucumber seedlings treated with CF from *P. simplicissimum* GP17-2 before and after challenge inoculation of *C. orbiculare* that was done 24 hrs after CF-treatment. Total RNA was isolated using guanidium isothiocyanate. Three μg total RNA was fractionated in a 1% agarose gel. After electrophoresis, the RNA was transferred to a nylon membrane. Hybridization was performed in 0.05M sodium hydrate solution with Digoxygenin-labeled probes of peroxidase, chitinase and β-1,3-glucanase at 55-65°C.

Results

Cucumber hypocotyls pretreated with the CFs from PGPF isolates showed a greater degree of lignin deposition 18 hrs after inoculation of *C. orbiculare* as compared to the control. The CFs of PGPF isolates generated H_2O_2 from cucumber fruit disks (Table 1). Maximum chemiluminescence emission was detected using the CF of *P. simplicissimum* GP17-2. Hypocotyls of cucumber seedlings expressed peroxidase and chitinase genes after 2 hrs of CF-treatment of *P. simplicissimum* GP17-2, while expression of β-1,3-glucanase gene was not observed even after 24 hrs of CF-treatment (Figure 1). When the hypocotyl of CF-treated cucumber was challenge inoculated with *C. orbiculare*, the activation of peroxidase and chitinase genes was more rapid and prominent, and β-1,3-glucanase gene expression was also observed.

Table 1. Lignin deposition on hypocotyls of cucumber seedlings induced by culture filtrate from PGPF isolates, following challenge inoculation with *Colletotrichu orbiculare*

Treatment	Hours after inoculation of *C. orbiculare*					
	3	6	12	18	24	48
Penicillium simplicissimum GP17-2	−	−	−	+	++	+++
Fusarium equiseti GF18-3	−	−	−	−	++	++
Phoma sp. GS8-2	−	−	−	−	++	++
Acibenzolar-S-metyl	−	−	−	+	+++	+++
SDW	−	−	−	−	+	+

The degree of lignin deposition was evaluated by counting the percentage of germinated spores with appressoria. About 100 spores were evaluated for each treatment. The values of 0%, < 30%, 31-70% and > 70% are presented as −, +, + + and + + +, respectively.

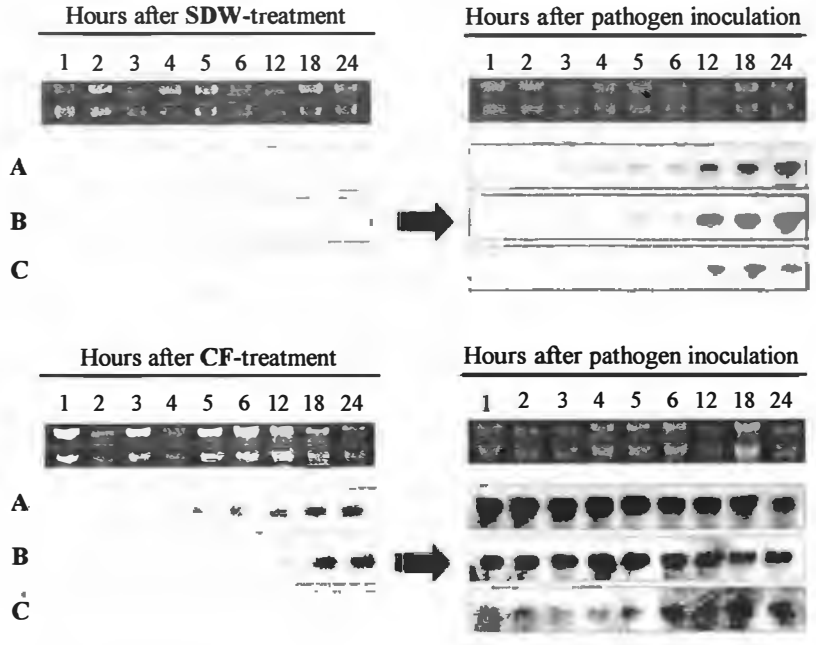

Figure 1. Northern blot analysis showing the expression of A) Peroxidase, B) Chitinase and C) β-1,3-glucanase mRNAs in cucumber hypocotyls at different time after treatment of culture filtrate from *Penicillium simplicissimum* GP17-2 and inoculation of *Colletotrichum orbiculare*

Discussion

Among CFs of PGPF isolates, CF of *P. simplicissimum* GP17-2 showed the most rapid and intensive lignin deposition on cucumber hypocotyls after inoculation of *C. orbiculare*. H_2O_2 generation is known as the first signal of defense response against pathogens. H_2O_2 was rapidly induced by CF of GP17-2. Expression of peroxidase gene increased within 24 hrs after treatment with CF of GP17-2. H_2O_2 produced in the cell wall could be used as a substrate by various peroxidases for lignin synthesis. From these results, the mechanism of the disease suppression induced by CF of GP17-2 could be associated with the strengthening of the host plant's cell wall.

The expression of pathogenesis related genes (peroxidase and chitinase) was detected in the cucumber hypocotyls treated with CF of GP17-2 before challenge inoculation of *C. orbiculare*, suggesting the occurrence of priming effect in the cucumber plants. After challenge inoculation, direct attack against pathogen by hydrolyzing enzyme activity like chitinase and β-1,3-glucanase could also be involved one of the mechanisms of induced resistance by CF of GP17-2.

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Mixture of two antagonists: Influence on expression of their key biocontrol factors

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Abstract: Introduction of bacterial and fungal biocontrol agents offers a promising alternative to manage soilborne diseases. The combination of a bacterial with a fungal antagonist could be a useful method to enhance biocontrol activity. Fluorescent pseudomonads are established biocontrol agents against several soilborne pathogens. Production of the polyketide 2,4-diacetylphloroglucinol (Phl) contributes substantially to their antimicrobial activity. The expression of the phlACBDE biosynthetic operon of strain CHA0 was measured by using a translational phlA'-'lacZ fusion. *Trichoderma* strains are well known for their biocontrol activity against several plant pathogens through chitinase production such as the ECH 42 endochitinase and the NAG1 N-acetyl-β-glucosaminidase. The *Trichoderma* strains used contain a fusion of the *Aspergillus niger* goxA reporter gene with either the ech42 or nag1 5' upstream noncoding sequences. The effect of the combination of *Pseudomonas fluorescens* CHA0 and *Trichoderma atroviride* P1 on the expression of important biocontrol genes of *T. atroviride* P1 and *P. fluorescens* CHA0 was studied. Phl enhanced nag1 expression, whereas an unidentified substance from *P. fluorescens* CHA0 repressed expression of both chitinases studied. Addition of *T. atroviride* P1 culture filtrates to the growing medium of *P. fluorescens* enhanced in the exponential growing phase β-galactosidase-activity, whereas in the early stationary phase no differences in gene expression was observed. These results indicate that negative and positive effects on expression of key biocontrol genes may occur while mixing antagonists.

Key words: *Trichoderma atroviride* P1, *Pseudomonas fluorescens* CHA0, gene expression, chitinase, PHL

Introduction

Concerns about performance reliability are a major impediment to large-scale application of biocontrol products. Application of a mixture of biocontrol agents and understanding the sources of variability in biocontrol activity could be important to enhance biocontrol activity. The polyketide metabolite 2,4-diacetylphloroglucinol (Phl) is one of the most effective antimicrobial metabolites produced by strains of fluorescent pseudomonads. Environmental factors and complex biotic factors, such as plant species, plant age, host cultivar, infection with the plant pathogen *Pythium ultimum* and the fungal toxin fusaric acid produced by *Fusarium oxysporum*, can significantly alter phlA expression (Notz et al., 2001; Notz et al., 2002). The expression of the phlACBDE biosynthetic operon of strain CHA0 was measured by using a translational phlA'-'lacZ fusion. The antagonistic fungus *Trichoderma atroviride* is a potential biocontrol agent against a wide range of aerial and soilborne plant pathogens. Its mycoparasitic activity may be due the production of several chitinolytic enzymes such as the ECH 42 endochitinase and an N-acetyl-β-glucosaminidase (NAG1) that are involved in mycoparsitism. The *Trichoderma* strains used contain a fusion of the *Aspergillus niger* goxA reporter gene with either the ech42 or nag1 5' upstream noncoding sequences.

Material and methods

Influence of secondary metabolites of* P. fluorescens *CHA0 on ech42 and nag1 expression in* T. atroviride *P1

One plug of a actively growing culture of *T. atroviride* P1 derivatives containing either the ech42-gox or the nag1-gox fusion was inoculated on a 1.5% malt agar plate amended with none, 2,4-diacetylphloroglucinol (Phl), pyoluteroin (Plt) or salicylic acid (Sal) respectively to a final concentration of 0.1 µM in the medium. After 66 hrs of growth at 24°C in the dark, glucose oxidase activity was measured as describe below.

Influence of* P. fluorescens *CHA0 derivatives on expression of key biocontrol genes of* T. atroviride *P1

One plug of *T. atroviride* P1 derivatives ech42 or nag1 was inoculated on a 1.5% malt agar plate. Overnight cultures of the P. fluorescens wild-type strain CHA0 and its derivatives CHA660 (Plt-neg.), CHA631 (Phl-neg.) or CHA89 (gacA-neg.) were co-inoculated with four spots of 20 µl in distance of 3.2 mm to the T. atroviride P1 plug. After 66 rs of growth at 24°C in the dark glucose oxidase activity was measured as describe below.

Measurement of glucose oxidase activity

10 ml of a phosphate-puffer was added to each plate. After rotary shaking (80 rpm) for 30 min, 100 µl of these aliquots were analyzed for glucose oxidase activity according to Mach et al. (1999).

***Influence of* T. atroviride *culture filtrate on phlA'-'lacZ expression* in vitro**

P. fluorescens CHA0 carrying a translational phlA'-'lacZ fusion on plasmid pME6259 were grown in 24 ml of King's medium B amended with 6 ml of fungal filtrates. 30 µl aliquots from an exponential growth-phase LB culture of the bacterial strain were used for inoculation. β-Galactosidase activities were determined throughout the exponential and stationary growth phase by the method of Miller (1992).

Expression of a phlA'-'lacZ fusion in* P. fluorescens *CHA0 on residues of two maize varieties

Maize leaves of the two varieties Corso and Magister were cut in pieces and surface sterilized. The leaves were soaked for 1.5 hrs in a bacterial suspension of strain CHA0 carrying a translational phlA'-'lacZ fusion. 5 g of the plant material was inoculated with one plug of an actively growing culture of *T. atroviride* wild-type strain P1. After incubation of one week at 24°C in the dark, 20 ml of phosphate-puffer was added to flasks and shacked for 10 min at 80 rpm. The liquid phase was assessed for β-Galactosidase activity according to Miller (1992).

Results

Secondary metabolites of* P. fluorescens *CHA0 alter expression of one key biocontrol gene in* T. atroviride *P1

Expression of both chitinase genes was not influenced by the secondary metabolites Plt and Sal amended to the media. In contrast, Phl enhanced more then 2-fold nag1-, but not ech42 expression.

Different* P. fluorescens *CHA0 derivatives alter ech42 and nag1 expression differentially

By co-inoculation of *T. atroviride* P1 with different derivatives of *P. fluorescens* on agar plates expression of both chitinases was repressed by 40% compared to the control. This repressing effect was absent for nag1 expression by co-inoculation with *P. fluorescens* CHA0 derivatives producing Phl.

Culture filtrates of* T. atroviride *P1 alter the expression of a phlA'-lacZ fusion in* P. fluorescens *strain CHA0

Addition of *T. atroviride* P1 culture filtrate to the growing media of *P. fluorescens* CHA0 enhanced phl'-'lacZ expression during exponential growing phase, whereas in the early stationary growing phase phlA expression was equal to the control treatment.

Expression of a phlA'-'lacZ fusion in strain CHA0 on crop residues of two different maize varieties co-inoculated with* T. atroviride *P1

The phl'-'lacZ fusion was expressed, when P. fluorescens was grown for one week on crop residues of two maize variety Corso and Magister. The co-inoculation with *T. atroviride* P1 repressed phl expression per CFU by 40% compared to the control on both maize varieties.

Discussion

By combination of antagonists, the expression of their key biocontrol factors may be altered. Neutral, enhancing and even repressing effects were found. Upon the selection of good biocontrol partners, it should be taken into account that due to repressing effects on expression of key biocontrol genes the performance of the antagonists may be reduced.

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Analysis of biocontrol-deficient mutants in *Pseudomonas chlororaphis* reveals genes involved in regulation of biocontrol

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Abstract: The regulation of biocontrol activity in isolates of *Pseudomonas chlororaphis*, efficient biocontrol agents of fungal, seed-borne diseases in barley, is largely unknown. Seeds infested with the net blotch pathogen (*Drechslera teres*) and subsequently coated with a suspension of the above bacteria produce healthy seedlings, whereas uncoated seeds produce seedlings with typical net blotch symptoms. One of the modes of action of the biocontrol agent is the production of an antifungal substance, DDR (2,3-deepoxy-2,3-didehydro-rhizoxin), a polyketide. DDR plays a role, but it is not the only mode of action. After random transposon (miniTn*5-uidA*) mutagenesis of an efficient bacterial isolate, mutants were screened for loss of biocontrol activity against *D. teres* on seeds. Genetic and phenotypic analysis of the eight biocontrol mutants with the lowest biocontrol efficacy uncovered additional biocontrol components for this bacterium, one of which was GacS, a sensor kinase which controls a variety of biocontrol traits, e.g., DDR production. The other mutated genes also appear to be involved in regulation of biocontrol activity, based on DNA sequence homology of mutated genes to regulatory genes and of simultaneous alterations in a variety of traits, such as motility and the production of fluorescent siderophores and extracellular protease.

Key words: biological control, antimicrobial, plant disease, biocontrol mechanisms

Introduction

The biocontrol agent *Pseudomonas chlororaphis* strain Vb10 (Borowicz, 1998) is a fluorescent pseudomonad that has high efficacy against seed-borne fungal diseases of cereals, in greenhouse and field trials. When seeds that are naturally infested with, for example, *Drechslera teres*, the net blotch pathogen, and coated with a log phase suspension of Vb10 cells grown in liquid culture, the emerging seedlings are free from infection. The conditions used in the greenhouse bioassay employed mimics those in field conditions (Hökeberg, 1998). Thus, when working with mutants or at times of the year that do not allow field experimentation, the greenhouse bioassay is very useful. Microorganisms used in biocontrol of plant pathogens generally utilize several modes of action or mechanisms. The most commonly encountered modes of action in the literature of fluorescent pseudomonads are colonization ability (Lugtenberg et al., 2001), the ability to produce antimicrobial compounds and HCN (Fenton et al., 1992; Blumer & Haas, 2000), competition against microorganisms e.g., through siderophore production (Mulya et al., 1996) and the ability to induce resistance in the plant (van Wees et al., 1997). At the outset of this study, we knew that an antifungal polyketide, named DDR (2,3-deepoxy-2,3-didehydrorhizoxin) (Svensson, 1999) was involved in disease suppression, and that it did not account for all of the biocontrol activity (Wright, unpublished data). The residual biocontrol activity of a DDR-deficient mutant was presumably due to other mechanisms. Our approach was to mutagenize strain Vb10 with a

transposon (miniTn5-uidA) and subject the transposon mutants to bio-tests, in order to uncover novel biocontrol traits, without bias for the production of antifungal compounds.

Materials and methods

P. chlororaphis strain Vb10 was randomly mutagenized with miniTn5-uidA. Kanamycin resistant colonies of Vb10: miniTn5-uidA were transferred to TSB (Tryptic soy broth, Difco, Detroit, MI, USA) supplemented with kanamycin and cultured for 48 hrs before cells were harvested and applied to barley seeds (cv. Svani), originating from a seed lot that was naturally infested with *D. teres*. The greenhouse bio-test was performed as described by Hökeberg (1998) for 600 miniTn5-uidA-mutants of Vb10, each pot containing 50 seeds and one pot per mutant. Forty mutants were tentatively assigned as "biocontrol defective", and they were screened once more, using 6 replicate pots of each mutant treatment. From this second screening, a subset of eight mutants with consistently lowered biocontrol efficacy emerged: Vb11, Vb12, Vb13, Vb14, Vb15, Vb16, Vb17 and Vb18. A third bio-test was performed, again using six replicates per treatment, this time, including all mutants in the same test. HPLC analysis of the culture supernatants of these eight mutants was performed in order to assess whether or not they produced DDR (J. Levenfors, personal communication).

The eight mutants were also characterized phenotypically with regard to production of extracellular protease and fluorescent colonies on King's medium B, and for motility in a swarming assay (Bondesson, 2002; Thorsson, 2002). Finally, six of the eight mutants (Vb13 through Vb18) were characterized genetically. A portion of genomic DNA adjacent to and containing the transposon insertion of each mutant was cloned in the pBCSK+ vector (Stratagene, La Jolla, CA, USA) and sequenced. The DNA sequences were edited, assembled and analyzed with DNA sequence software (DNAStar, Lasergene, Madison, WI, USA) and through BLAST searches (Altschul et al., 1997).

Results and discussion

After the second bio-test screening, the eight mutants were ranked according to descending biocontrol efficacy relative to the positive and negative controls in each test in the following manner: Vb16, Vb11, Vb12, Vb15, Vb17, Vb18, Vb13 and Vb14. Results of the third bio-test agreed with the second test in the relative ranking of the mutants, although the percent infection was much lower and thus it was impossible to accurately assess the efficacy of the mutants that were the least impaired in biocontrol efficacy (i.e., Vb11, Vb12, Vb15, Vb16 and Vb17). Also mutants Vb13, Vb14 and Vb18 protected better than in bio-test 2. There was thus some factor(s) in the environmental conditions that differed between bio-tests 2 and 3 that enhanced the biocontrol efficacy of many mutants.

Mutants Vb13 and Vb14 were the least effective in biocontrol. They had lost their DDR and protease production, and swarming ability, but they displayed enhanced fluorescence on King's B (Table 1). They were mutated in GacS, a membrane-bound sensor kinase that is known to control a variety of cellular functions and thereby biocontrol (Laville et al., 1992) in other biocontrol systems, and pathogenicity in pathogenic pseudomonads (Heeb & Haas, 2001), in response to environmental stimuli.

Mutant Vb18 was interesting in that the transposon also in it seemed to have knocked out a membrane protein (or two). The transposon insertion has inserted in a gene that is adjacent and in the same operon as another membrane protein. The regulatory role of these/this protein(s) is apparent from the results of phenotype tests (Table 1). The two genes have homology to genes in *P. aeruginosa* with unknown functions.

The remaining mutants did not have a striking biocontrol phenotype in the third bio-test, but did so in bio-test 2. They also appear to be regulatory mutants, and it is possible therefore, that the absence of one biocontrol trait could be overcompensated for by the augmented presence of another. For instance, under the conditions of the third test, the cultures were shaken at lower speed and without as much aeration. This could have increased DDR production to a level higher than in the second bio-test, thus masking the biocontrol effect of the absence of any other biocontrol mechanism.

Table 1. Genes mutated, their putative functions and the phenotypes conferred by the mutations of "biocontrol defective mutants"

Bacterial strain	Homolog to mutated gene	Putative functions	Phenotypes				
			Bio-test 3 (% inf)	DDR	Fluorescence	Swarming	Protease
Vb10wt			0*	+	+	+	+
Vb13	gacS	global regulation	34	-	+++	-	-
Vb14	gacS	global regulation	37	-	++	-	-
Vb18	In *P. aerug.* genome	regulation/ membrane protein?	16	-	-	+++	++
Vb15	crfX	sigma-factor, OprF	2	+	+	+	+
Vb16	In *P. aerug.* genome	NifR3 family protein- regulation?	2	+	+	+	+
Vb17	In *P. aerug.* genome	Signal peptide/ exported protein?	0	+	+	++	++
Vb12	N.D.	-	2	+	+	+	+
Vb11	N.D.	-	0	+	+	+	+

*The results of bio-test 3 are indicated as percentage plants with net blotch symptoms on the first leaf, as compared to the control treatment with TSB-treated seeds (=100%). N.D. = not determined.

In summary, we have confirmed work by others (Laville et al., 1992; Heeb & Haas, 2001) that GacS is important for effective biocontrol also in strain Vb10 as it is in other fluorescent pseudomonads. We have found that GacS regulates DDR production. In addition, we have uncovered novel regulatory genes whose involvement in cellular regulation is so far unknown.

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Biological activity of the rust antagonist *Cladosporium tenuissimum* Cooke and its secondary metabolites

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Abstract: The hyperparasite *Cladosporium tenuissimum* sporulate and produce a large amount of conidia when cultivated on different C/N ratio media. The capability of this hyperparasite to establish intimate contact with the bean rust agent *Uromyces appendiculatus* in vitro and to reduce symptom expression where tested in planta. Ethyl acetate crude extracts from large scale cultures of *C. tenuissimum* contained five chemically related metabolites, which were purified and characterized by spectral data analysis. The main compound resulted to be cladosporol 1, $C_{20}H_{16}O_6$, a known decaketide inhibitor of the glucan biosynthesis. Metabolites 2-5 are first described and derivatives of 1. Major compounds 1-3 were tested at concentrations between 12.5 and 100 ppm as inhibitors of bean rust uredospore germination and mycelial growth of several phytopathogenic fungi. Positive results are discussed on the basis of chemical features and concentration: the activity was especially significant against *Oomycota*. Difference in sensitivity was displayed by the other fungi, some of which being more sensitive to metabolite 2, which has a further oxidation on C-4. Compounds 1 and 2 were always more active than 3, which lacks the epoxide moiety in the molecule compared with them. Cladosporol, investigated in planta as potential therapeutic compound in the host/pathogen *U. appendiculatus* / *Phaseolus vulgaris* system, showed a fairly good inhibitory activity when given simultaneously to rust inoculation. Protective efficacy decreased in preventive treatments, likely due to the easy degradability of the compound. On the basis of obtained data, the potential use of the fungus and its metabolites as biocontrol therapeutic agents is discussed.

Key words: *Cladosporium tenuissimum*, secondary metabolites, cladosporol, biological control, *Uromyces appendiculatus*, phytopathogenic fungi

Introduction

Cladosporium tenuissimum Cooke has biocontrol activity against the pine stem rust agent *Cronartium flaccidum* and its related mycrocyclic form *Peridermium pini*, and seems to play a key role in decreasing the amount of the disease in Italian natural wood ecosystem (Moricca et al., 2001). The selected strain ITT21 was investigated for studying the variety of host specificity and deepening the multiple biocontrol mechanisms exerted by the fungus.

Materials and methods

Conidia production on different substrates

A fungal suspension of *C. tenuissimum* (0.5 ml) was inoculated in 10 Petri dishes of four rich semisynthetic media (A-D), whose composition is listed in Table 1. After 14-days, conidia

were withdrawn by washing plates twice with 0.01% Tween 20 (5 ml), filtered on a gauze double layer, counted by Kova slides, and freeze dried.

Production of secondary metabolites

The same four media A-D were used for large scale 14-day growth of *C. tenuissimum* (40 Roux flasks each) and extracted with organic solvent (ethyl acetate + 1% methanol). Crude extracts were purified by chromatography techniques (silica gel columns with a stepwise elution with dichloromethane/methanol and PLC in suitable eluents) until purity of the single compounds, which were weighted. Chemical characterization was reached by 1H and 13C NMR, MS, IR, UV spectra on the pure compounds and their derivatives obtained by chemical reactions.

Growth inhibition of some phytopathogenic fungi

Mycelial disks (0.7 cm diameter) of the 13 fungi listed in Table 2 were inoculated on PDA Petri plates, amended with metabolites 1-3 at four concentrations (12.5, 25, 50 and 100 µg/ml). Percent of growth inhibition was measured at 3, 6 and 10 days by comparison with controls (media without metabolite). Tetraconazole (5 µg/ml) represented a positive standard. Water agar slides, amended with metabolites 1-3 at the four concentrations, were seeded with uredospores collected from infected bean plants and resuspended in 0.01% Tween 20 (5 x 105 spores/ml; 25 µl). Slides were maintained at 21°C in a moist Petri chamber for 24 hrs. Germination was calculated as % inhibition in comparison with control on water agar slides.

***Interaction between* U. appendiculatus *and* C. tenuissimum**

Three assays were performed by inoculating the two fungi on water agar slides as follows: A) host and antagonist simultaneously; B) host 24 hrs before antagonist; C) antagonist 24 hrs before host. Fungal spores ($5x10^5$/ml) suspended in 0.01% Tween 20 were inoculated (25 µl) at the selected time. Slides were observed by optical and SE microscopy at regular intervals starting three hours later till 96 hrs.

In planta assays

Conidia of ITT21 strain and cladosporol at the four concentrations were also tested as protective agents on bean plants (Borlotto nano cv."Lingua di fuoco", Ingegnoli) inoculated at the stage of primary leaves with *U. appendiculatus*. Plants were treated with conidia or cladosporol preventively, simultaneously and sub-sequentially to inoculation of the rust agent.

Results and discussion

C. tenuissimum strain ITT21 profusely sporulated on all selected rich semi-synthetic A-D media, reaching the maximum production of conidia after two weeks. Emergence of the conidiophore from the mycelium was detectable after about 3 days and it was unaffected by light requirements. As for other sugar fungi, sporulation was not related to exhaustion of a key nutrient and accompanied a rapid vegetative growth on all media. Growth of *C. tenuissimum* occurred on laminarin as a sole carbon source in vitro, but not on chitin.

Crude extracts purified by column chromatography enabled to recover at least 5 metabolites approximately constituting the 60% of total weight. Metabolite production was affected quantitatively, but not qualitatively by substrate composition. Table 1 indicates that medium D is the most effective for obtaining either the greatest amount of viable conidia or the larger production of metabolites. By spectral data analysis, the main compound resulted to be cladosporol 1, $C_{20}H_{16}O_6$, a decaketide already isolated from *Cladosporium cladosporioides* and known as glucan biosynthesis inhibitor and plant growth regulator (Sakagami et al., 1995). Metabolites 2-5 are derivatives of 1 and first described. All they contain a 2-tetralone chromophore bound through C-4' and C-8 (Figure 1). Compared with 1, metabolite 2 shows an oxidation on C-4, metabolite 3 lacks the epoxide group, metabolite 4 has an –OH instead

the epoxide group and metabolite 5 has a double hydroxylation on C-2 and C-3 instead the epoxide group (Nasini et al., unpublished data).

Table 1. Production of conidia and secondary metabolites by *Cladosporium tenuissimum* ITT21 strain after two-week cultivation on four different substrates

Media[a]	Number conidia/ml	mg conidia/100 ml substrate (d.w.)	Crude extract[b]	Metab. 1c	Metab. 2c	Metab. 3c	Metab. 4 c	Metab. 5 c
A	7.3×10^6	5.6	32	9.7	3.0	2.5	1.7	2.3
B	8.4×10^6	7.2	85	16.3	5.1	3.6	3.0	2.6
C	3.9×10^6	N.D.	22	5.2	3.4	1.8	0.9	1.3
D	1.6×10^7	40.3	179	39.4	24.3	17.2	16.4	9.1

[a]Medium A (malt extract, peptone, glucose and agar 20-2-20-15 g/L); medium B (A + glycerol 10 g/L); medium C (rice flour and agar 9-2 g/flask); medium D (corn steep liquor, glucose, saccharose, yeast extract, K_2HPO_4, agar 10-90-100-5-2-15 g/L). [b]Crude extract is referred as milligrams in weight from 100 ml of medium (one flask) after washing with hexane and is the mean obtained from 40 flasks. [c]Milligrams purified by the crude extract.

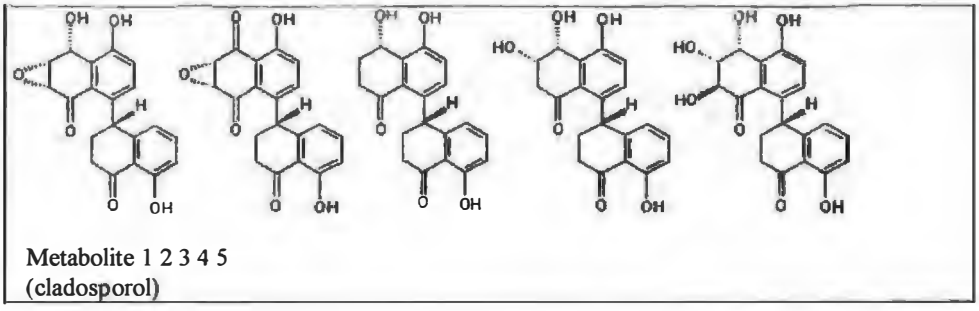

Figure 1. Metabolites 1-5 chemically characterized

Growth inhibition of 13 phytopathogenic fungi by the metabolites 1-3 at four concentrations and tetraconazole is reported in Table 2. Tetraconazole, as inhibitor of sterol biosynthesis, is more active on higher fungi than on Oomycota. ITT21 metabolites were especially effective on the four Phytophorae, whose growth was 100% inhibited by all three at 100 ppm. Other fungi were less sensitive, showing an inhibition up to 50% only in some cases. The activity against Oomycota could be explained as inhibition of 1,3-β-glucans of the cell wall as already showed for cladosporol by Sakagami et al. (1995).

In preventive treatments of bean plants, cladosporol, applied 24 hrs before inoculation of *U. appendiculatus* at 100 μg/ml reached a Protection Index of 19%. In treatments performed simultaneously with inoculation of rust, the Protection Index was 39, 20, 12 and 5% respectively at the four concentrations: these results indicate that the metabolite undergoes an easy degradation, which could be a good feature for natural compounds involved in biocontrol. *C. tenuissimum* conidia on bean inoculated with *U. appendiculatus* decreased disease symptoms down to 67 and 76%, respectively in simultaneous and preventive (24 hrs before) treatments, but they were inactive in curative ones. In water agar slides conidia

germinated later than uredospores. By optical and SE microscopy observations, an intimate contact between antagonist and host was showed by growth and coiling of hyphae of the mycoparasite over and through the rust spores (Saracchi & Maffi, personal communication).

The capability of compounds 1-3 to inhibit *U. appendiculatus* spore germination *in vitro* was related to concentration (Table 3). Rust spores showed a 79.1±0.8 germination percentage in the control. Metabolite 2 was the most active, since germination was completely inhibited at 100 and 50 ppm, significantly reduced to more than 90% at 25 ppm and still low at 12.5 ppm. Metabolite 1 (cladosporol) was more active than 3, reaching an inhibition value higher than 80% at the highest concentration.

Table 2. Growth inhibition of some phytopathogenic fungi by metabolites 1-3 in comparison with the IBS tetraconazole (5 μg/ml)

	Metabolite 1 (ppm)				Metabolite 2 (ppm)				Metabolite 3 (ppm)				IBS
Fungus	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	5
Alternaria alternata	55	45	33	26	45	53	34	31	41	35	28	23	60
Botrytis cinerea	19	14	12	10	28	23	7	4	32	25	6	4	67
Cercospora beticola	56	46	38	26	32	28	15	11	22	28	10	7	97
C. herpotrichoides	32	20	10	8	33	27	18	18	32	19	11	4	100
C. lindemuthianum	38	26	18	13	35	30	20	20	28	15	9	6	15
Fusarium roseum	36	21	17	8	49	40	15	9	43	28	15	13	55
H. teres	100	100	91	86	48	42	33	29	61	45	22	15	80
Mucor sp.	23	11	6	3	31	22	19	15	37	17	10	0	5
Rhizoctonia solani	35	29	22	10	45	43	28	26	44	27	15	15	52
Phytophtora capsici	100	56	43	37	100	60	49	23	63	46	29	17	-
P. cinnamomi	100	65	50	50	100	50	41	41	62	50	38	18	6
P. erytroseptica	100	100	100	62	100	61	39	36	100	70	52	24	15
P. nicotianae	100	75	68	45	100	48	40	23	100	70	48	23	3

Table 3. Inhibition % of *U. appendiculatus* spore germination induced by metabolites 1-3 at various concentrations (μg/mg) of substrate

Metabolite	100	50	25	12.5
1	84,2	56,1	25,9	10,5
2	100	100	92,1	84,9
3	77,6	50,9	12,7	10,5

Results seem to indicate that *C. tenuissimum* is able to parasitize more than one rust fungus and to interact also with Oomycota. Further investigations will be focused to better evaluate the potential spectrum of pathogens affected by this biocontrol agent and to develop formulations to improve effective approach in crop protection.

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Antagonistic effect of volatile and non volatile antibiotics produced by fungi isolated from apple phyllosphere on *Venturia inaequalis* (Cke.) Wint.

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Abstract: In this study, after 5 periodical isolations from apple leaves 30 different fungal isolates were determined on apple cultivars, Granny Smith, Starkspur Golden and Starkrimson, which had various susceptibility to *Venturia inaequalis*; a causal agent of apple scab. Some of these isolates were identified as *Cryptococcus* (white yeast), *Sporobolomyces* (pink yeast), *Alternaria, Penicillium, Cladosporium, Epicoccum, Heterosporium, Papularia, Peyronellaea* and *Papularia* on genus base. When the number of microorganisms/cm^2 leaf area was calculated, *Aurebasidium* sp. and Cryptococcus sp. were found as the major part of fungal population. Effects of volatile and non volatile antibiotics produced by mycoflora isolates *in vitro* on mycelial growth of *V. inaequalis* were examined. It is observed that volatile antibiotics produced in vitro by white and pink yeasts (*Cryptococcus* and *Sporobolomyces* sp.) inhibit colony development of the pathogen by 100% while their non-volatile antibiotics have no important inhibitory effect. Volatile antibiotics from *Alternaria* spp. (except A14), *Cladosporium* spp. (A16 and A34), *Penicillium* sp. (A11), *Epicoccum* spp. and *Papularia* sp. appeared as most efficient isolates by inhibiting mycelial development of *V. inaequalis* by 100%. Same effect was not observed with their non-volatile antibiotics. Among all isolates, non-volatile antibiotics from *Penicillium* sp. (A8) and *Papularia* sp. were the only ones with a complete inhibitory effect.

Key words: *Venturia inaequalis*, saprophyte mycoflora, antibiosis

Introduction

The most important disease of apples in Turkey is apple scab (*Venturia inaequalis* (Cke.) Wint.). This disease is important in other apple production areas of the world as well. Spay applications are timed with the guidance of forecast and warning systems with appreciable success; these systems are based on temperature and humidity data (Yürüt et al., 1988). However, problems associated with the use of pesticide in agricultural promoted the interest in biocontrol methods. Saprophytic microorganisms that colonize plant surfaces may compete with the pathogens on nutrients and space; some of these microorganisms limit the growth and development of the pathogens by producing volatile and non-volatile antibiotics. Also, some saprophytes may interact with the pathogens and inhibit their development by blocking the pathogens' hyphae and spore production. In addition, the saprophytic microorganisms may have a positive effect on plant development as they stimulate phytoalexin production or produce hormones-like gibberellins (Fokkema, 1976). In this study the effects of volatile and non-volatile antibiotics, produced *in vitro* by micoflora isolated from apple leaves, was tested against mycelia growth of *V. inaequalis*.

Material and method

Detection of apple phyllosphere micoflora

Saprophytic micoflora were sampled from leaves of apple varieties Granny Smith, Starkspur Golden and Starkrimson, which have different sensitivity against *V. inaequalis*. Sampling was done 5 times, from mid- April to late May. Leaf samples (4 g) were shaken in 100 ml sterile distilled water for 30 min at 180 rpm and 0.1 ml was taken from the suspension obtained by dilution at a rate of 10^{-2}, then homogeneously spread over PDA medium. Fungal population was calculated at the end of incubation period by counting fungus colonies that show differences. Then, these colonies were identified as genus or species after purification (Simmons, 1998; Rotem, 1994). *V. inaequalis* was isolated from infected fruits.

Production of volatile and non-volatile antibiotics by saprophyte fungus

Since antibiotics may be distributed in water, air and among other microorganisms, direct contact between two microorganisms is not necessary (Özaktan & Bora, 1998). In order to determine effect of volatile antibiotics produced by saprophytic fungal flora against mycelia growth of *V. inaequalis*, firstly, 6 mm diameter mycelia discs were cut from pathogen and saprophyte cultures, then inoculated to the centers of PDA Petri dishes. After removing the lids, these saprophyte and pathogen Petri dishes were placed upside-down over pathogen Petri dishes and then plate edges were tightly covered with parafilm (Dennis & Webster, 1971a). Control Petri dishes were prepared by covering PDA Petri plates with pathogen plates as above. These plates were incubated at 24°C for a month. At the end of this period, antagonistic effect was determined by measuring *V. inaequalis* colony diameters.

Cellophane membrane technique was used for determination of the effect of non-volatile antibiotics of saprophytic fungal flora against mycelia development of the pathogen. For this purpose, 9 cm diameter cellophane was sterilized and a pair was placed over the solidified surface of PDA medium in Petri plates. Cellophane membrane is permeable to the necessary nutrition elements for the fungus and non-permeable to spores, hyphal fragments and other structures. Petri dishes prepared by these methods were left for incubation after inoculating a mycelia disc from saprophyte cultures to the centers of Petri plates. Saprophyte fungus was taken from medium surface together with cellophane membrane before covering the Petri surface; and a mycelia disc of *V. inaequalis* was inoculated to the center of PDA medium. The same process was also applied for control Petri plates but this time saprophytic fungus discs were not placed (Dennis & Webster, 1971b). At the end of incubation period, antagonistic effect was determined again.

Results and discussion

Phyllosphere microbial population consists of species that might be in competitive relations to each other. In this study, white and pink yeasts (*Cryptococcus* and *Sporobolomyces* sp.), *Aurebasidium, Alternaria, Cladosporium, Penicillium, Epicoccum, Heterosporium, Papularia* and *Peyronellaea* species were isolated from apple phyllosphere micoflora. Blakeman (1981), reported that filamentous fungi that colonized on apple leaf surfaces are *Aurebasidium pullulans, Alternaria* spp., *Cladosporium* spp. and *Epicoccum* spp. A steady increase over time was obtained in phyllosphere microbial population, especially for the yeast population. This may be explained by the increase of substrates rich in KH (pollen, aphids, etc) that encourages yeast development at advanced periods of vegetation. Van Der Burg (1974) reported that leaves infected with aphids have high density of *Sporobolomyces* spp. Effectiveness of volatile and non-volatile antibiotics produced by saprophytic micoflora against mycelia development of pathogen is given in Table 1.

While volatile antibiotics produced by white and pink yeasts *in vitro*, inhibited development of *V. inaequalis* by 100%, effects of non-volatile antibiotics were not the same. Similarly, volatile antibiotics from *Alternaria* spp. (except A14), *Cladosporium* spp. (A16 and A34), *Penicillium* sp. (A11), *Epicoccum* spp. and *Papularia* sp. inhibited mycelia development of *V. inaequalis* by 100% but the efficacy of the non-volatile antibiotics was lower. Non-volatile antibiotics produced by *Penicillium* spp. (A8) and *Papularia* sp. were highly effective as well (100% efficacy) and these were the most effective among all other isolates that produce non-volatile antibiotics. Yeasts had no important inhibitory effect. Blakeman & Fokkema (1982) reported that pink yeasts could produce some antibiotics that were not effective in inhibition of the growth of *Cochliobolus sativus*.

Table 1. Reduction of mycelia growth (%) of *Venturia inaequalis* by volatile and non-volatile antibiotics produced by various fungi isolates

Fungi	Isolates No.	Volatile antibiotics	Non-volatile antibiotics
Cryptococcus sp.	A21	100.0	15.0
Sporobolomyces sp.	A3	100.0	28.2
Aurebasidium sp.	A18	19.0	38.5
Alternaria sp.	A22	35.3	2.6
A. alternata	A14	47.6	45.0
	A17	100.0	25.0
	A29	100.0	7.9
	A46	100.0	5.0
	A49	100.0	15.0
	A51	100.0	20.0
Cladosporium spp.	A16	100.0	25.0
	A34	100.0	13.2
C. herbarum	A33	9.5	30.0
	A41	17.6	40.0
Penicillium spp.	A7	35.0	47.5
	A8	40.0	100.0
	A9	35.0	48.7
	A11	100.0	64.1
	A12	19.0	55.0
	A48	41.2	20.0
Epicoccum spp.	A10	100.0	51.3
	A24	100.0	21.1
	A30	100.0	7.9
	A39	100.0	15.8
	A42	100.0	25.0
Heterosporium sp.	A13	42.9	66.7
Papularia sp.	A1	100.0	100.0
Peyronellaea spp.	A25	35.3	18.4
	A31	47.6	10.5
	A52	14.3	20.0

Production of antibiosis is just one of the various mechanisms by which microorganisms can inhibit pathogenic fungi. Saprophytic fungi may fail to inhibit the development of

pathogen *in vitro*, but it does not mean that they would necessarily found ineffective in nature. Efficacy in nature is affected by the microorganisms, the host plants and the environment and *in vitro* tests do not reflect the complexity saprophyte-pathogen interactions in as nature. Accordingly, although yeast and *Cladosporium* spp. has no antagonistic effect against the pathogen *in vitro*, they may effectively suppress the pathogen *in vivo* (Redmond et al., 1987).

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In vitro antifungal activity of *Trichoderma harzianum*, *T. longibrachiatum*, *T. asperellum* and *T. atroviride* against *Botrytis cinerea* pathogenic to strawberry

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Abstract: *Trichoderma* species have been investigated as biocontrol agents for over 70 years due to the ability of several *Trichoderma* strains to antagonize other filamentous fungi, including many plant pathogenic species. Phylogenetic analysis based on ITS1 and ITS2 sequences have clustered biocontrol strains of *Trichoderma* into four groups: *T. harzianum* - *T. inhamatum* species, *T. longibrachiatum*, *T. asperellum* and *T. atroviride* - *T. koningii* complex. This study evaluates the *in vitro* antifungal activity of extracellular proteins (CWDEs) produced by 20 biocontrol strains of *Trichoderma* belonging to four different ITS-based species. Crude preparations were obtained by ammonium sulphate precipitation (90% saturation) of culture filtrates after growing the fungi 4 days in a minimal media supplemented with an appropriate carbon source (glucose 5 g/L and chitin 20 g/L). Culture filtrates were concentrated 75-fold and stored at –20ºC before use. Antifungal assays were performed including potato dextrose broth, conidial suspension and enzymatic extract in microwell plates. After 12 hrs, the inhibition of hyphae growth of the strawberry pathogen *Botrytis cinerea* was determined in presence of the tested enzyme solution (300 ppm). Sterile bi-distilled water was used as negative control for these assays. *T. asperellum* 25, *T. atroviride* 26, *T. atroviride* 33, *T. inhamatum* 51 and *T. asperellum* 53 showed the highest index of antifungal activity. Therefore, the most effective biocontrol isolates against *B. cinerea* belonged to three different ITS-based species. These results demonstrate that there is not a species-related antifungal activity among the biocontrol strains considered in the present study.

Keywords: CWDEs, antifungal activity, *Trichoderma*, *Botrytis cinerea*

Introduction

Trichoderma strains have been widely used as biocontrol organisms in agriculture, due to their ubiquity and rapid substrate colonization (Grondona et al., 1997), their capacity to synthesize substances inducing SAR (systemic acquired resistance) in plants (Enkerli et al., 1999), their potential for promoting plant growth (Inbar et al., 1994), the inhibitory effect of their antibiotics (Sivasithamparam et al., 1998; Keszler et al., 1998) and their capability of producing cell wall degrading enzymes (CWDEs) (Lorito et al., 1998) against many plant pathogens. Phylogenetic analysis based on ITS1 and ITS2 sequences have clustered biocontrol strains of *Trichoderma* into four groups (Hermosa et al., 2000): *T. harzianum* - *T. inhamatum* species, *T. longibrachiatum*, *T. asperellum* and *T. atroviride* - *T. koningii* complex. However, most of the strains used as biocontrol agents belong to *T. harzianum*.

This study evaluates *in vitro* antifungal activity of extracellular proteins produced by 20 biocontrol strains of *Trichoderma* belonging to four different ITS-based species, against the

strawberry pathogen *Botrytis cinerea*. The aim was to elucidate if there is a relationship between a specific molecular taxonomic group and its antifungal activity.

Material and methods

Fungal material

Strains of *Trichoderma* were obtained from several sources as indicated in Table 1. Strain 98 of *B. cinerea*, isolated from strawberry, was used as target fungi in the *in vitro* bioassays.

Table 1. *Trichoderma* strains used in this work

Ref. No.	Collection number	Species	Geographic origin
36	IMI 293162	*T. inhamatum*	India
24	IMI 352940	*T. inhamatum*	Spain
41	IMI 298374	*T. inhamatum*	United Kingdom
51		*T. inhamatum*	Asia
37	IMI 296235	*T. harzianum*	Colombia
66		*T. harzianum*	Brazil
49		*T. harzianum*	Brazil
34		*T. harzianum*	USA
39		*T. harzianum*	United Kingdom
44	IMI 304058	*T. longibrachiatum*	India
68		*T. longibrachiatum*	Brazil
3	IMI 20179	*T. asperellum*	France
25	IMI 296237	*T. asperellum*	Colombia
53	IMI 20268	*T. asperellum*	Spain
7	IMI 224801	*T. asperellum*	India
4	IMI 131883	*T. viride*	United Kingdom
50		*T. viride*	Chek Republic
11	IMI 352941	*T. atroviride*	France
33	IMI 352939	*T. atroviride*	United Kingdom
26	IMI 238904	*T. atroviride*	United Kingdom

Enzyme production

For the production of extracellular enzymes, *Trichoderma* isolates was grown in 250 mL Erlenmeyer flasks containing 100 mL of a synthetic medium with chitin and glucose as carbon sources, composed of 15 g of KH_2PO_4, 5 g of glucose, 20 g of chitin, 0.59 g of $MgSO_4.7H_2O$, 0.60 g of $CaCl_2\ 2H_2O$, 1 g of $(NH_4)_2SO_4$, 0.005 mg of $FeSO_4.7H_2O$, 0.0016 mg of $MnSO_4\ H_2O$, 0.0014 mg of $ZnSO_4.7H_2O$ and 0.002 mg of $CoCl_2$ in 1 L of distilled water, finally adjusted to pH5.5. Culture filtrates were concentrated 75-fold by ammonium sulphate precipitation (90% saturation), dyalized against bidistilled water and stored at –20°C before use.

Antifungal assays

Antifungal assays included potato dextrose broth, a conidial suspension of *B. cinerea* and extracellular proteins of *Trichoderma* at a concentration of 300 ppm in microwell plates. After 12 hrs of incubation at 25°C in darkness, hyphae growth was determined in presence of the tested protein solution. Sterile bi-distilled water was used as negative control for the assays. The area of each well covered by micellium was measured with the program Q-Win (Leica Microsystems) modified for this particular application.

Results and discussion

CWDEs produced by the complete set of twenty strains were used in antifungal assays. Eleven strains (*T. inhamatum* 36, *T. inhamatum* 24, *T. inhamatum* 51, *T. asperellum* 53, *T. asperellum* 25, *T. harzianum* 34, *T. harzianum* 39, *T. viride* 50, *T. atroviride* 11, *T. atroviride* 33 and *T. atroviride* 26) showed significant differences in their antifungal activity against *B. cinerea* (Figure 1).

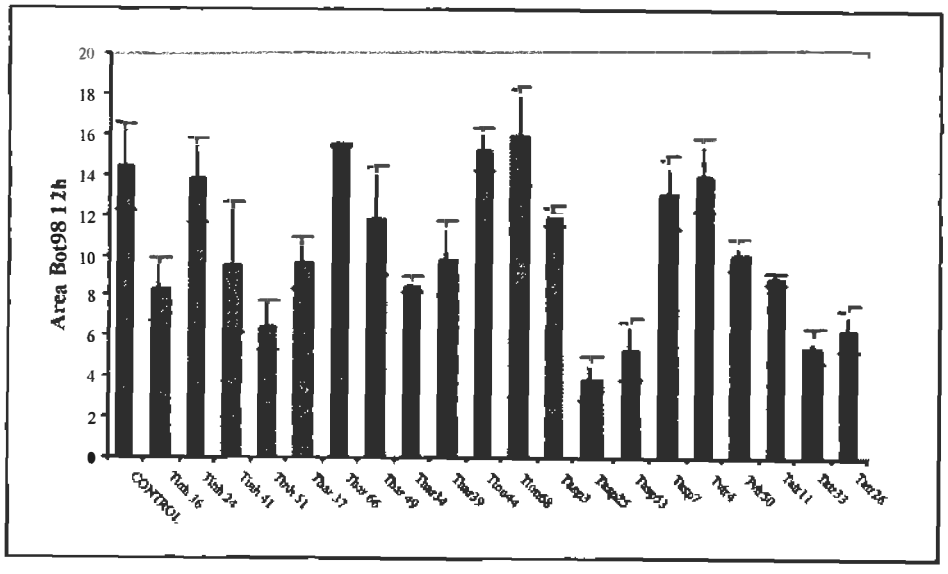

Figure 1. Effect of dyalized extracellular proteins of *Trichoderma* (at concentration of 300 ppm) on hyphae growth of *Botrytis cinerea*

Trichoderma strains 51, 53, 25, 33 and 26 presented the highest index of antifungal activity, reducing the hyphae growth of *B. cinerea* by more than 50% (Figure 2). Therefore, the most effective biocontrol isolates belonged to three different ITS-based species. These results demonstrate that there is not a species-related antifungal activity among the biocontrol strains considered in the present study.

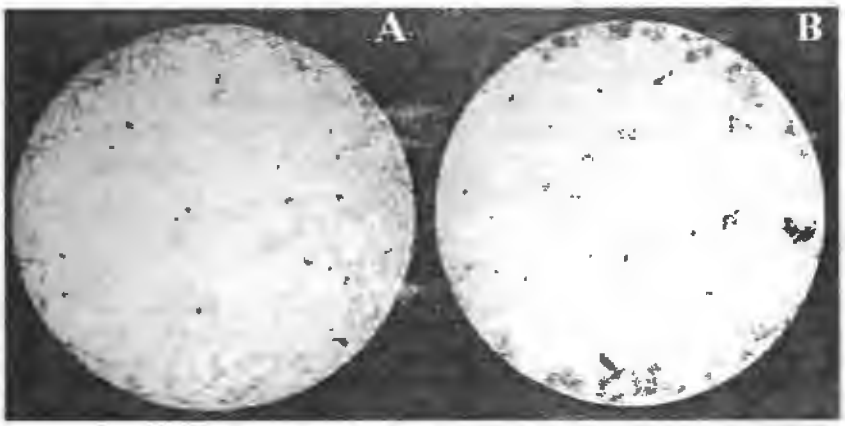

Figure 2. Hyphae growth of *B. cinerea* 98 in presence of sterile bi-distilled water after 12 hrs (A); and inhibition of hyphae growth of *B. cinerea* 98 in presence of *T. asperellum* 25 protein solution at 300 ppm after 12 hrs (B)

Acknowledgements

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Trichoderma protein ability in preventing grey mould and anthracnose diseases on strawberry leaves and petioles

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Abstract: The effectiveness of *Trichoderma* spp. protein solutions in preventing grey mould and anthracnose disease on strawberry was examined. The assays have tested enzyme solutions on leaf discs and petioles of strawberry against disease agents, *Botrytis cinerea* and *Colletotrichum acutatum*, respectively. In addition, the antifungal activity was studied when the proteins were mixed with different supports. The results indicate that some of the protein solutions reduced the development of *B. cinerea* on strawberry leaves and the combination of the proteins with commercial plant coatings can promote and inactivate the antifungal action of both pathogens.

Key words: biological control, *Trichoderma* spp., grey mould, anthracnose agent, protein solutions

Introduction

Grey mould and anthracnose diseases, caused by *Botrytis cinerea* and *Colletotrichum acutatum* respectively, are responsible for large economical losses in strawberry (*Fragaria x ananassa*) crops. Biocontrol represents a suitable alternative to control these pathogens. Mycoparasitic strains of the genus *Trichoderma* are very well known biocontrol agents due to their antagonistic abilities. In this study, we have tested *Trichoderma* spp. enzyme solutions on strawberry plant leaves and petioles against grey mould and anthracnose agent, respectively. The antifungal activity was also assayed when the proteins were mixed with different supports.

Material and methods

Fungal material

The four *Trichoderma* strains studied correspond to the following species (Hermosa et al., 2000): *T. harzianum* 37 (Tharz 37), *T. harzianum* 59 (Tharz 59), *T. inhamatum* 41 (Tinha 41) and *T. virtde* 63 (Tvir 63). *Trichoderma* spp. were grown and maintained on potato dextrose agar (PDA, Sigma). The pathogen strains, *B. cinerea* and *C. acutatum*, obtained from strawberry, were also grown and maintained on PDA.

Enzyme production

The liquid induction media consisted of a minimal salt media supplemented with natural chitin (Patagonian crabshell), with the pH adjusted to 5.5. Liquid media (100 ml) were inoculated with 10^5 conidia per ml and incubated for 4 days in shacked flasks, at 28°C.

Culture filtrates were concentrated 35-fold by ammonium sulphate precipitation (90% saturation), dialyzed against bi-distilled water and stored at –20°C before use.

Supports for protein application

Enzyme solutions were applied on vegetal material, alone or in combination with different supports: a pine resine (2% v/v VaporGard, agrichem Co.) or two wetters (0.02% v/v Agral or 1% v/v Canplus, Zeneca Co.).

***Antifungal assays on leaf discs of strawberry against* Botrytis cinerea**

The assays were performed on 9 mm-diameter leaf discs of strawberry cv. Aromas, following a modification of the method of Peng & Sutton (1991). The preventive treatment consisted in submerging the discs in protein solutions (400 ppm), alone or with a support. Control discs were submerged in sterile distilled water instead of protein. Each support was also applied without protein. Fifty minutes after preventive treatment, one thousand conidial were placed on the surface of each disc. Twenty eight discs were prepared by treatment. Once the conidia had germinated, the discs were incubated on paraquat-chloramphenicol agar plates at 20-22°C for eleven days. Then, the incidence and the severity indexes were noted down for each disc. The incidence index corresponds to the number of discs with conidiophores. The severity scale was based on the percentage of disc area which showed conidiophores and spores of *B. cinerea*: 0 (0%), 1 (1-10%), 2 (11-50%), 3 (51-75%) and 4 (76-100%).

***Antifungal assays on detached petioles of strawberry against* Colletotrichum acutatum**

The protein solutions produced by the strain Tharz 59 were confronted with conidia of *C. acutatum*, following a modification of the methods of O'Neill et al. (1996) and Freeman et al. (2001). In these antifungal assays, the youngest end of petiole portions (5 cm) was treated with 10 µl of protein solution. The infection was carried out with 1,200 spores of *C. acutatum* and was let to develop for 10 days at 22°C and 100% relative moisture, in the dark.

Previous studies showed that the protein solutions produced by Tharz 59 strain were the only ones that totally prevented the spore germination and the hyphae growth of these phytopathogen.

Results and discussion

***Antifungal assays on leaf discs of strawberry against* Botrytis cinerea**

The percentage of discs that showed sporulation of *B. cinerea* was 100% in all cases, except discs treated with the Tharz59 protein mixed with VaporGard which showed only 61% of the discs infected. Severity indexes obtained in antifungal assays on leaf discs of strawberry are shown in Figures 2-4. The combination of the Tharz 59 protein solution with VaporGard reduced the severity index in 63%, in comparison with the control. On the other hand, mixing of each protein solution with Canplus wetter showed results similar to the control (data not shown). In relation to the Agral wetter, it promoted itself the reduction of severity indexes in 18%, and this reduction was maintained in combination with Tharz 37 or Tharz 63 protein solutions, in comparison with control treatment.

***Antifungal assays on detached petioles of strawberry against* Colletotrichum acutatum**

The percentage of petioles that showed antracnose lesions was 100% in all cases. The lesions corresponding to petioles treated with Tharz 59 protein solution and infected with spores of *C. acutatum* are shown in Figure 5. Ten days after infection with *C. acutatum*, the combination of the Tharz 59 protein solution with VaporGard allowed the reduction of *C. acutatum* lesion by 67%. However, the mixing of Tharz 59 protein solution with Agral or Canplus did not imply a decrease in lesion length.

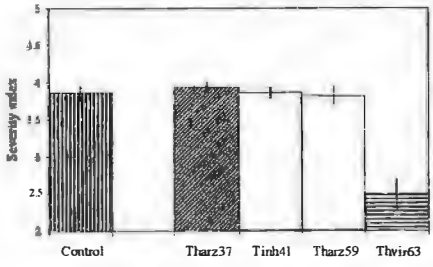

Figure 1. Severity indexes of *B. cinerea* on leaf discs of strawberry treated with protein solution (400 ppm).

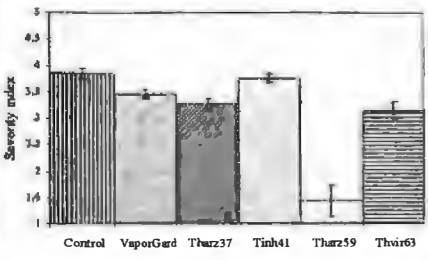

Figure 2. Severity indexes of *B. cinerea* on leaf discs of strawberry treated with protein solution (400 ppm) mixed with VaporGard

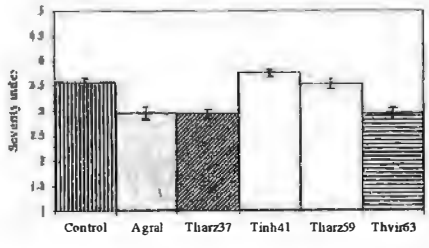

Figure 3. Severity indexes of *B. cinerea* on leaf discs of strawberry treated with protein solution (400 ppm) mixed with Agral

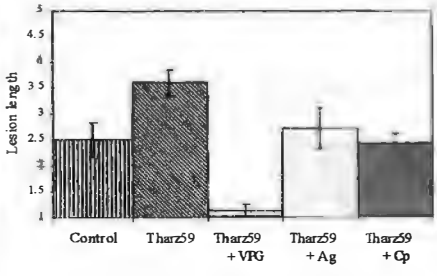

Figure 4. Lesion length (cm) in detached petioles of strawberry treated water (control) or Tharz 59 solution (mixed or not with a support), and also infected with conidia of *C. acutatum*

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Enhancement of some plant resistance to formae sp. *Fusarium oxysporum* by chitosan

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Abstract: Amendment of potato-dextrose agar with microcrystaline chitosan even at 1000 µg/ml only slightly inhibited linear growth of *Fusarium oxysporum*. The compound, however, suppressed the development of *F. oxysporum* f. sp. *dianthi* in peat. Drenching of carnation with chitosan at concentrations of 500 or 1000 µg/ml effectively controlled *Fusarium* wilt development and decreased the spread of the pathogen in plant vessels. Chitosan applied as soak of gladiolus corm and bulb of tulip already at 100 µg/ml suppressed the development of *Fusarium* rot symptoms.

Key words: *Fusarium oxysporum*, chitosan, carnation, gladiolus, tulip, wilt, rot, inhibition

Introduction

Formae speciales of *Fusarium oxysporum* still cause widespread, often heavy economic losses in commercially grown ornamental plants. *F. oxysporum* f. sp. *cyclaminis* and f. sp. *dianthi* are the most dangerous threats of cyclamen and carnation wilt whereas *F. oxysporum* f. sp. *gladioli* and f.sp. *tulipae* are the causal agents of corm and bulb rot (Orlikowski, 1987) Disinfection of soil or substrata, cultural practices, treatment of substratum and plant materials with fungicides are the most often used strategies for controlling of pathogens. Another potential approach involves the use of chitosan, which is β-1, 4-glucosamine polymer. The compound inhibits fungal growth and activates defence mechanisms of plants towards some pathogens (Stossel & Leuba,1984; Wojdyła, 2001). Chitosan also can induce a multitude of biological processes in plant tissues, including the stimulation of chitinases, accumulation of phytoalexins, synthesis of proteinase inhibitors and increased lignification (El Ghaouth et al., 1994). Application of chitosan enhances resistance of tomato to *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Benhamou & Theriault, 1992), controlled root rot of cucumber incited by *Pythium aphanidermatum* and triggered several host defence responses, including the induction of structural barriers in root tissues and the stimulation of antifungal hydrolases (El Ghaouth et al., 1994). The purposes of this research were to determine the effect of chitosan on *Fusarium oxysporum* growth and on the development of *Fusarium* wilt and *Fusarium* rot on tested plants; and to determine the influence of the compound on *F. oxysporum* population dynamic in substratum.

Materials and methods

Chitosan and fungi

Microcrystaline compound derived from crab-shell chitin with Mw=70000, obtained from Institute of Chemical Fibre in Łódź, Poland, was used in all trials.. *Fusarium oxysporum* Schlecht. f. sp. *cyclaminis* Gerlach (*Foc*), *F. oxysporum* Schlecht. f. sp. *dianthi* (Prill. et Del.) Sny. et Hans. (*Fod*), *F. oxysporum* Schlecht. f. sp. *gladioli* (Massee) Sny. et Hans. (*Fog*) and

F. oxysporum f. sp. *tulipae* Apt (*Fot*) were used. Stock cultures were maintained on potato-dextrose agar (PDA) at 24°C in the dark. For peat infestation *Fod* was prepared on Quick oat.

In vitro *trials*

Influence of chitosan on linear growth of *Foc* and *Fod* was evaluated on PDA. Five mm diameter mycelia disks, taken from the rim of 7-day-old cultures, were placed in centre of 90 mm Petri dishes filled with the medium amended with chitosan at doses from 0 to 1000 µg/ml. After 5 and 7-day-incubation at 24°C the diameter of the colonies was measured.

***Influence of chitosan on population dynamic of* F. oxysporum *f. sp.* Dianthi**

One dm^3 pots were filled with peat artificially infested with *Fod* and carnations were planted. Immediately after planting, chitosan at concentrations of from 500 to 1000 µg/ml (50 ml of solution/pot) was applied. Pots were placed on greenhouse bench and incubated at 13-22°C. Number of colony forming units (cfu) was determined before planting of carnation (initial population) and 4, 10 and 12 weeks after the peat was amendment with the compound using Komada (1975) selective medium.

Influence of chitosan on carnation, gladiolus and tulip health

Development of *Fusarium* wilt symptoms on carnation was observed 8, 10 and 14 weeks after planting. Additionally, discoloration of vessels was observed. In protection of gladiolus and tulip, chitosan was used as corms and the bulbs were soaked 24, 48 hrs before, and 24 hrs after, inoculation with 2 formae speciales of *F. oxysporum*. Development of necrosis on plant materials was observed 12 (tulip) and 14 days (gladiolus) after inoculation.

Experimental design was completely randomised with 4 replications and 1 Petri dish, 10 cuttings, corms or bulbs in each rep. Trials were repeated at least twice.

Results

Growth of formae sp.* Fusarium oxysporum *in the presence of chitosan

Observation of linear growth of 3 f.sp. *F. oxysporum* showed that chitosan only slightly inhibited their development (Figure 1). Fungal growth was not suppressed when chitosan was added to the medium at doses ranging from 1.6 to 8 µg/ml. However, even in the highest concentration tested (1000 µg/ml) inhibition of growth did not exceed 22 % (Figure 1). Effects of the various chitosan concentrations on growth of the three formae specials of *F. oxysporum,* were insignificant.

Influence of chitosan on population dynamic of Fusarium oxysporum f. sp. dianthi

Application of chitosan at dose of 500 µg/ml to peat infested with 660 cfu of the pathogen/g, resulted in only slight, non-significant increase in population density within 4-weeks of incubation (Figure 2). In the next 8 weeks, population density fluctuated between 610 and 750 cfu/g. Increase of chitosan dose to 1000 µg/ml resulted initially in a significant decrease in pathogen population density; however, during the next 2 months population density in that treatment was similar to that recorded in the lower concentration of the compound (Figure 2). In a non-treated substratum, a 2.5-fold increase in pathogen density was noted within the first 10 weeks. After that time, population density decreased (Figure 2).

Influence of chitosan on healthiness of carnation, gladiolus and tulip

Application of chitosan to the peat medium immediately after carnation planting resulted in significant inhibition of *Fusarium* wilt development during the 14-weeks of growth. Differences in disease intensity between the two chitosan doses used were insignificant (Figure 3). Analyse of vessels discoloration of carnations treated once with chitosan at doses 500 and 1000 µg/ml, showed that the compound significantly inhibited the spread of the pathogen within the plants (Figure 4). Drenching of plants with the compound at

concentration of 500 µg/ml resulted in the most pronounced decrease of vessels discoloration (Figure 4).

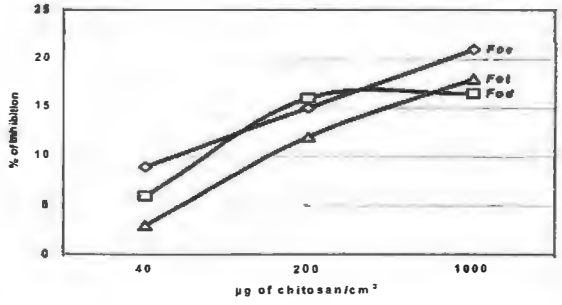

Figure 1. Growth of formae sp. *Fusarium oxysporum* in the presence of chitosan

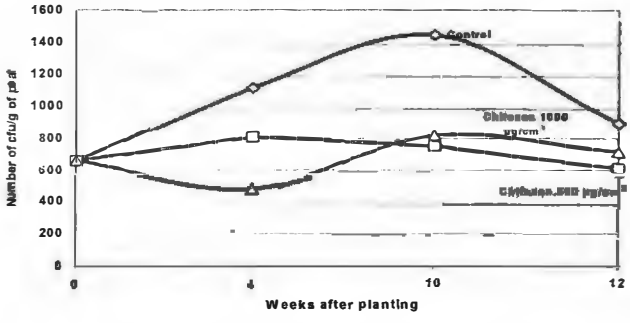

Figure 2. Relationship between chitosan concentration, incubation time and population density of *Fusarium oxysporum* f. sp. *dianthi* in peat

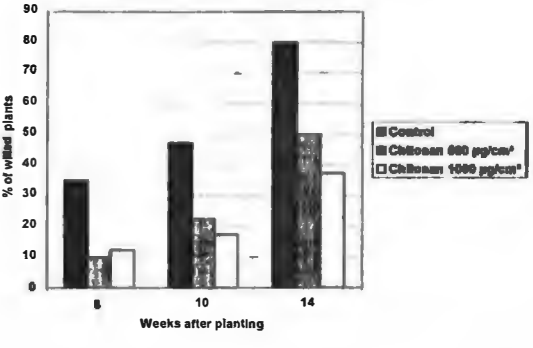

Figure 3. Activity of chitosan applied as peat drench in the control of *Fusarium* wilt of carnation

Soaking of gladiolus corms in chitosan solution 24 or 48 hrs before inoculation with *Fog* suppressed the development of corm rot for up to 52% (Figure 5). The compound used at 500 µg/ml 24 hrs before inoculation of corms was significantly more effective than the higher doses used. Application of the compound 24 hrs after inoculation resulted in almost complete inhibition of the *Fusarium* rot development (Figure 5). Soaking tulip bulbs in chitosan solution (at concentration of 2500 µg/ml) 24, 48 or 72 hrs after inoculation with *Fot*, inhibited the development of *Fusarium* rot for about 50% (Table 1). Soaking of bulbs in solution of 100 µg chitosan/ml 24 hrs after inoculation, suppressed the disease for at least 72%. Effectiveness of the compound in that concentration decreased significantly when applied 48 or 72 hrs after inoculation (Table 1).

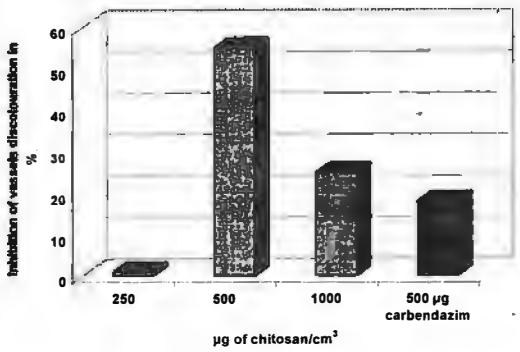

Figure 4. Influence of chitosan on the spread of *Fusarium oxysporum* f.sp. *dianthi* in carnation vessels

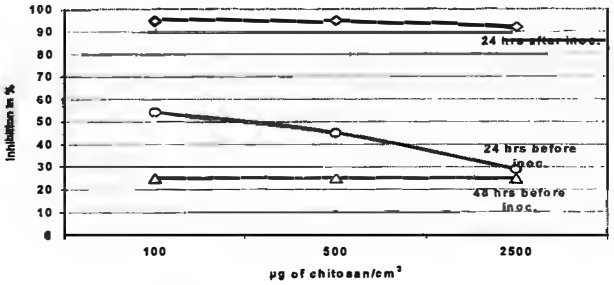

Figure 5. Relationship between chitosan concentration, inoculation time and development of *Fusarium* corm rot of gladiolus; inhibition of necrosis spread in %

Table 1. Relationship between chitosan concentration, application time and development of *Fusarium* rot of tulip bulbs; inhibition of necrosis spread in % 12 days after inoculation

Treatment	µg/cm³	Hours before inoculation of bulbs		
		24	28	72
Chitosan	100	75 b	49 a	33 a
Chitosan	500	72 b	62 b	54 b
Chitosan	2500	42 a	55 ab	55 b

Means in columns, followed by the same letter, do not differ with 5% of significance; Duncan's multiple range test.

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Production of salicylic acid and pseudomonine and suppression of disease by *Pseudomonas fluorescens* WCS374

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Abstract: Siderophore production by fluorescent *Pseudomonas* spp. is involved in control of soil-borne plant pathogens. It has been suggested that both competition for iron and induction of systemic resistance (ISR) are the effective mechanisms of siderophore-mediated disease suppression. *Pseudomonas. fluorescens* WCS374 suppresses fusarium wilt in radish by ISR, however, in *Arabidopsis thaliana* the strain can not induce resistance. WCS374 produces the siderophore pseudobactin and salicylic acid (SA) at low iron availability, and it has been suggested that both compounds are involved in ISR in radish. After Tn*5* insertion mutagenesis of strain WCS374, several pseudobactin deficient mutants were obtained. These mutants still produce a siderophore, the production of which is linked to SA biosynthesis. We cloned a region of the WCS374 genome that contains the loci necessary for SA and the second siderophore (pseudomonine) production, and part of this clone has been sequenced. Mass spectrometric data obtained from pseudomonine reveal that a SA moiety is present in the molecule, linked to cyclothreonine and a histamine group. We speculate that in some plant pathogen systems WCS374 is not effective because it does not produce SA but instead it produces pseudomonine. We investigated effects of growth conditions on SA and pseudomonine production in WCS374 in relation to suppression of disease.

Key words: *Fusarium oxysporum*, induced systemic resistance, ISR, *Pseudomonas fluorescens*, *Pseudomonas syringae* pv. *tomato*, salicylic acid, siderophores

Introduction

Siderophores are produced by microorganisms under conditions of low iron availability. The production of siderophores by certain strains of fluorescent *Pseudomonas* spp. has been linked to suppression of soil-borne diseases (Bakker et al., 1986; Buysens et al., 1996; Duijff et al., 1999; Handelsman & Stabb, 1996). It has been suggested that siderophores are antagonistic by means of sequestering iron from the environment, restricting growth of the pathogen (Lemanceau et al., 1992; Loper & Buyer, 1991). Siderophores have also been implicated in inducing ISR in plants (Leeman et al., 1996; Van Loon et al., 1998). Salicylic acid is another compound that can be produced by bacteria under conditions of low iron availability and application of SA to plants can induce a systemic resistance. ISR triggered by *P. aeruginosa* 7NSK2 in bean against *Botrytis cinerea* was suggested to be due to the production of SA by this bacterium (De Meyer & Hofte, 1997). *P. fluorescens* WCS374 can produce large amounts of SA under low iron availability, and it was suggested that SA produced by this bacterium is involved in ISR against fusarium wilt in radish (Leeman et al., 1996). In *Arabidopsis thaliana*, WCS374 can not induce resistance, despite the fact that application of SA to this plant species induces resistance (Van Wees et al., 1997). To find an explanation for these observations we analysed a gene cluster involved in the biosynthesis of SA in WCS374 and discovered that WCS374 produces a second siderophore,

pseudomonine, that contains a SA group (Mercado-Blanco et al., 2001). Effects of growth conditions on biosynthesis of SA by WCS374 were investigated in the present study.

Material and methods

Influence of histidine on SA production by WCS374

The non fluorescent siderophore pseudomonine produced by WCS374 contains a SA moiety linked to cyclothreonine and a histamine group. Since the pseudomonine biosynthetic gene cluster contains a histidine decarboxylase gene (Mercado-Blanco et al., 2001), SA and pseudomonine production in WCS374 may be influenced by feeding with histidine. WCS374 was grown in standard succinate medium (SSM) containing a range of concentrations of histidine for 48 hrs and SA production was determined as described by Leeman et al. (1996).

Influence of temperature on SA production by WCS374

The fluorescent pseudobactin type siderophore is not produced by WCS374 at 37°C, whereas at this high temperature pseudomonine is produced. Since the production of SA and pseudomonine in this bacterium are linked the influence of incubation temperature on SA production was investigated. WCS374 was grown in SSM at 28, 31 or 33°C for 48 hrs and SA production was determined.

ISR in* A. thaliana *by WCS374 grown at different temperatures

An ISR bioassay with *P. syringae* pv. *tomato* DC3000 was performed according to the method described by Pieterse et al. (1998). WCS374 was grown on Kings medium B agar plates at 28 and 33°C for 24 hrs, and washed cells were suspended and used as inducing bacteria in the ISR bioassay.

Results and discussion

Addition of histidine to the growth medium drastically reduced the production of SA by *P. fluorescens* WCS374 (Table 1). At a concentration of 2.5 mg/ml histidine reduced SA production to a level around the detection limit.

Table 1. Influence of histidine on SA production by WCS374 grown in SSM medium

Histidine (mg/ml SSM medium)	SA production (fg/cell)
0	50
1	30
2.5	8
5	4
10	6
15	3

When grown at 31 or 33°C, WCS374 produced more SA than at 28°C in SSM medium (Table 2). We speculate that more pseudomonine, and at the same time also more SA, are produced to compensate for the lack of pseudobactin production at these higher temperatures.

Table 2. SA production by WCS374 grown in SSM at 28, 31 or 33°C

Incubation temperature (°C)	SA production (fg/cell)
28	19
31	27
33	26

In the ISR bioassay, WCS374 grown at 28°C did not reduce disease symptoms caused by *P. syringae* pv. *tomato*, confirming results of Van Wees et al. (1997). However, when grown at 33°C a significant reduction in disease symptoms by WCS374 was observed (data not shown) indicating that growth conditions of the bacteria can influence their performance as biocontrol agents. We speculate that WCS374 grown at lower temperatures does not induce ISR in *Arabidopsis* because it does not produce SA in the rhizosphere but pseudomonine due to the presence of histidine. When grown at elevated temperature WCS374 produces more SA and it can induce ISR in *A. thaliana*. We are currently investigating the possible involvement of both pseudomonine and SA in the observed disease suppression in the ISR bioassay.

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Control of soilborne diseases of spinach and tomato using fermented products of *Carica papaya*

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Abstract: Fermented products of papaya (FPP) were tested for their ability to control soilborne diseases like fusarium wilt in spinach (caused by *Fusarium oxysporum* f.sp. *spinaciae*) and tomato (caused by *F. oxy.* f.sp. *lycopersici)*. In spinach, soaking seeds with 0.05% FPP was effective to enhance plant stand of spinach. Drenching seedlings planted in artificially infested soil with 0.01% FPP, reduced disease severity and vascular discoloration significantly for 28 days after transplanting. In tomato, seed and seedling treatments with FPP significantly reduced fusarium wilt for 60 days after inoculation. In plants treated with 0.01% and 0.05% FPP protections of both, disease severity and vascular discoloration severity, was significant. Pathogen population in the roots of spinach and stems of tomato treated with FPP was significantly lower than that of control. As FPP does not have any antibiotic activity, the inhibition of pathogen multiplication could be related to induced systemic resistance in the plants.

Key words: soilborne diseases, fermented plant product, induced systemic resistance

Introduction

Fermented papaya product (FPP) is a natural Japanese health supplementation manufactured by natural fermentation of the crude juice of *Carica papaya*. FPP has been known to have several biological activities including free-radical regulating, immune-modulating and metal ion-chelating properties. Osato et al. (1995) indicated that FPP can regulate active oxygen species (AOS) in living cells of mammals.

In plant cells, Prusky (1988) and Elad (1992) reported that several antioxidants controlled both necrotrophic and biotrophic fungal pathogens in several crops, chiefly by negating the effects of free-radicals produced during pathogenesis. On the contrary, AOS produced in response to pathogens and elicitors has been hypothesized to have direct antimicrobial effects and may play a role in induced resistance. In this way, free-radicals and antioxidants have complex effects on plant diseases. It is known that FPP has dual functions, scavenging and generating of AOS, in mammalian system, which brought interest to study whether there is an effect on plant diseases. We demonstrated previously that treatment of cucumber seeds with FPP could induce systemic resistance against anthracnose caused by *Colletotrichum orbiculare* (Mondal et al., 2000). The objectives of this study were to determine the effect of FPP on suppression of Fusarium wilt in spinach and tomato and to study the defense mechanisms induced by FPP using a model of cucumber-anthracnose system.

Materials and methods

Hosts and pathogens

The spinach cv. Okame and the tomato cv. Momotaro were used throughout the study. *Fusarium oxysporum* f.sp. *spinaciae* (FOS) and *Fusarium oxysporum* f.sp. *lycopersici* (FOL) were used as Fusarium wilt pathogens of spinach and tomato.

Assay of FPP for control of Fusarium wilt of spinach

FPP (Sun-O-International, Inc., Gifu, Japan) was dissolved in distilled water at different concentrations. As for the seed treatment, seeds were soaked with FPP solutions and incubated at 25°C for 24 hrs. The seeds were directly sown into the soil artificially infested with barley grain inoclum (0.2%, w/w). As for the seedling treatment, seeds were sown into potting soil contained in paper pots. The 15-day-old seedlings grown in paper pots were treated with a solution of FPP (0.01%) at 10 ml/pot 3 days before transplanting. The treated seedlings were transplanted into the artificially infested soil with spore suspension of FOS at a density 10^5spores/g soil. The plants were grown at 25°C for 30 days in growth chambers with a 10 and 14 h light (7,500 lux)/ dark period. Disease severity based on the foliar symptom and discoloration severity of vascular tissue/cortex/xylem on the basal stem or crown were assessed using a scale of 0 to 4 (0: healthy, 1: yellowing, 2: slight wilting, 3: severe wilting, 4: dead plant) and 0 to 3 (0: no discoloration, 1: ≤1/3, 2: 1/3～2/3, 3: ≥2/3 of discolored vascular area), respectively.

Assay of FPP for control of Fusarium wilt of tomato in hydroponics rock wool system

This experiment was tested in hydroponics system using cubes of rock wool substrates in two size, mini (3.7x3.7x4.0 cm) and large (7.5x7.5x6.5 cm). The seeds were soaked with FPP solution and incubated at 25°C for 24 hrs. Seeds were sown into mini cubes and grown for 30 days in a growth chamber at 25°C. One mini cube containing one seedling was inserted into a large cube and drenched with 200 ml of solution of FPP (0.01 or 0.05%). After 10 days, 200 ml of spore suspension ($1x10^5$ cells/ml) of FOL was inoculated in each large cube. The plants were kept in the greenhouse for 100 days. Disease severity based on foliar symptom of wilting, which was monitored for 20 to 60 days after inoculation of pathogen, and vascular discoloration severity were assessed using same scale as mentioned above.

Results and discussion

Spinach seeds soaked in FPP for 24 hrs at 25°C showed significantly higher plant stand than water soaked seeds in artificially infested soil (Table 1). Suppression of foliar wilt and vascular discoloration by FPP persisted throughout the period studied. Seed treatment with 0.05% FPP was the most effective. In seedling treated with 0.01% FPP, reduction of disease severity and vascular discoloration severity in plants during 14 to 28 days after transplanting was 70 to 93% and 74 to 100%, respectively (Figure 1). The population of FOS in soil contained inside paper pots and in roots of spinach treated with a solution of FPP was significantly less than that of control (Figure 2).

In tomato, seed and seedling treatments with FPP solutions reduced Fusarium wilt significantly for 60 days after inoculation. Effects of FPP on both disease severity and vascular discoloration severity in plants were significant; disease severity was reduced by 76 and 53% and vascular discoloration severity was reduced by 55 and 50%, when treated with FPP at 0.01 and 0.05%, respectively. Also, the pathogen population in the stems of tomato treated with FPP was significantly lower.

Table 1. Plant stand of spinach (%) grown in artificially inoculated soil with barley grain inocula (0.2%, w/w) of *Fusarium oxysporum* f.sp. *spinaciae* after soaking seeds into solutions of FPP

FPP concentration (%, w/v)	Days after sowing		
	14	21	28
0.1	35.0 bc*	25.1 B	10.0 b
0.05	43.3 c	25.1 B	18.3 c
0.02	33.3 bc	13.2 Ab	11.7 b
0.01	31.7 bc	22.2 B	15.0 bc
0.005	20.9 b	16.7 Ab	10.0 b
Control	8.3 a	10.0 A	0.0 a

*Values are mean of two trials, each of 6 replicates. Mean values having the same letter do not differ significantly ($P \leq 0.05$) according to Duncan's multiple range test.

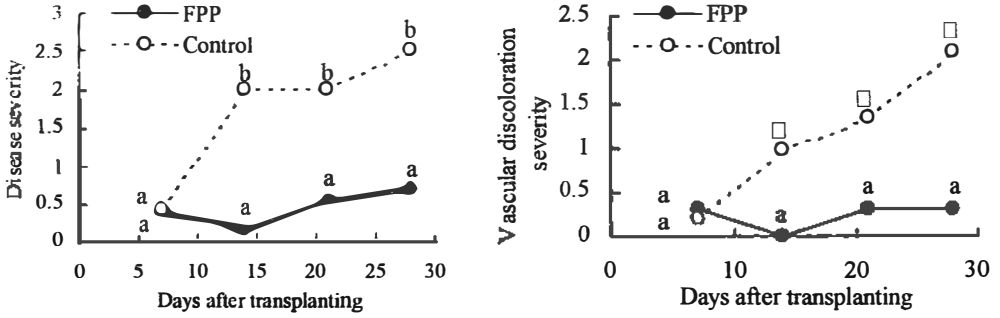

Figure 1. Disease severity and vascular discoloration severity of spinach grown in artificially infested soil with 10^5 spores/ g soil of *Fusarium oxysporum* f.sp. *spinaciae* after treatment of seedlings with FPP (0.01%). Treatments marked by a common letter do not differ significantly (P=0.05) according to Duncan's multiple range test.

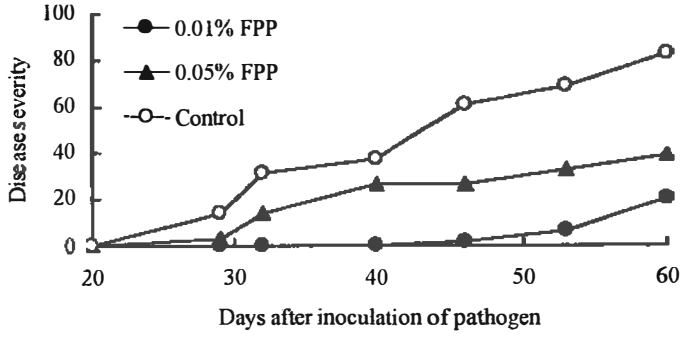

Figure 2. Disease progression of Fusarium wilt based on foliar symptom of wilting on tomato treated with FPP under rock wool hydroponics system

Thus, FPP treatments not only suppressed disease severity but also the size of pathogen population in spinach and tomato. As FPP does not show any antibiotic activity, this inhibition of pathogen multiplication could be related to induced systemic resistance in plants. We demonstrated previously that treatment of cucumber seeds with FPP induced systemic resistance against anthracnose caused by *Colletotrichum orbiculare*. There are many reports concerning induced systemic resistance using beneficial microbes, avirulent pathogen and pathogen or their metabolites and certain chemicals. In addition, the use of plant extracts from leaves of *Reynoutria sachalinensis* F. Schmidt (Nakai) has been proposed to prevent infection of cucumber by powdery mildew (Daayf et al., 1995). Our information of induced systemic resistance in plants using fermented plant products like FPP is novel in plant system. This result suggests that there is possibility of finding additional kinds of inducers from fermented plant products in the future.

In the model of cucumber-anthracnose system, no difference in peroxidase activity between FPP-treated and non-treated plants was observed before and after pathogen inoculation. In native-PAGE, the fast moving anodic peroxidase isozymes were enhanced gradually after challenge inoculation but no differences could be indicated in both treatments. Furthermore, FPP did not induce lignification at points where was penetrated by the pathogen. Therefore, the mechanism involved in resistance induced by FPP might be different with the one of pathogen induced systemic acquired resistance. The amount of tannic acid and ascorbic acid in spinach treated with FPP increased and antioxidant potential of the plant was raised (Kagawa & Inari, personal communication). It seems that the behavior of these antioxidants in the plant was probably involved in the mechanisms of disease control by FPP. Further research is needed to elucidate the mechanisms responsible for the activity of induced resistance in plants induced by FPP.

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The gelatinous matrix of *Meloidogyne* spp.: protection against egg parasites

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Abstract: The antimicrobial properties of the gelatinous matrix of *Meloidogyne incognita* against 3 nematophagous *V. chlamydosporium* isolates and a *V. dahliae* strain were explored in *in vitro* studies. TSB medium was inoculated with $1X10^3$ conidia of each test organism to which 4 *M. incognita* egg masses were added. After seven days of incubation, all fungi showed an almost entire reduction of growth compared to control treatments without egg masses. In a second experiment, egg masses were placed on cellophane paper in the centre of a Petri dish. After 4 days of incubation, the cellophane paper was removed and the *Verticillium* isolates were placed in the centre of the dish. This resulted in more than 60% growth reduction of all fungi after 10 days of incubation. The results were different for *V. chlamydosporium* isolate that was originally isolated from a *Meloidogyne* egg mass, where the growth was only inhibited by 20%. The results show that the *Meloidogyne* gelatinous matrix has antimicrobial properties, inhibiting nematophagous fungi to enter and to parasitize the eggs. This first report also shows that fungi originally isolated from egg masses seem to be more resistant to these substances, but further research including more isolates has to be performed.

Key words: egg mass, bacteria, *Meloidogyne, Verticillium chlamydosporium*

Introduction

Meloidogyne root-knot nematodes cause considerable damages to plants all over the world, in Europe mainly in greenhouses. Because of the phase-out of methyl bromide, a very effective and commonly used nematicide, biocontrol of plant-parasitic nematodes has become increasingly important (Csinos et al., 2000). The facultative parasite of root-knot nematodes *Verticillium chlamydosporium* is considered to have potential as a biocontrol agent against these pests because it is able to colonize the plant roots and parasitize the nematode eggs (De Leij & Kerry, 1991). However, the effectiveness to control nematodes differs significantly between different *V. chlamydosporium* isolates.

To reach the nematode eggs, the antagonist has to enter the egg mass first, into which the eggs of *Meloidogyne* species are deposited. This gelatinous matrix is thought to have several crucial functions in the life cycle of the nematode. The matrix is involved in lysing the host tissue in which the female nematode is embedded forming a canal from the posterior end of the nematode to the gall surface through which the eggs can reach the root surface (Orion et al., 1987). However, since the egg mass remains intact in the soil without being consumed by the surrounding soil micro-organisms, another function that is postulated is protection of the root-knot nematode eggs against soil microflora. Papert & Kok (2000) demonstrated that the egg mass of *M. hapla* is a densely populated niche with a distinct bacterial population. *In vitro* experiments also showed a significant reduction of a number of rhizosphere bacteria in the presence of *M. javanica* egg masses while other bacteria were not inhibited at all (Orion &

Kritzman, 1991). In this paper the antimicrobial properties of the *M. incognita* gelatinous matrix were explored in two *in vitro* studies.

Material and methods

Fungal isolates and media

The following nematophagous isolates of *V. chlamydosporium* were used: Vcc600.88 originally isolated from cysts of *H. avenae* (CBS collection, Baarn), Vc10 isolated from *Meloidogyne* egg masses (IACR-Rothamsted) and Vc0083 isolated from nematode infested soil (CLO, Merelbeke). In previous *in vitro* studies, these isolates had proven to be parasitizing eggs efficiently. The *V. dahliae* strain was isolated from infected tomatoes (KU Leuven, Belgium). Fungi were grown at 22°C and routinely sub-cultured on potato dextrose agar plates.

Assay using microcentrifuge tubes

Mycelium of every test isolate was scraped off from one month old PDA culture plates and dissolved in sterile water. The suspension was poured over glass wool and the conidia in the filtrate were counted under a light microscope. Microcentrifuge tubes were filled with 900 µl of Tryptone Soya Broth and 100 µl of spore suspension of each isolate containing $1X10^3$ spores. Four *M. incognita* egg masses were added to each tube in 3 replications, no egg masses were added to controls. The tubes were shaked for 7 days at room temperature. At day 0, 4 and 7, 100 µl was taken out of each tube, a dilution series was made and 100 µl of each dilution was plated on PDA enriched with chloramphenicol (100 mg/l). After 4 days of incubation, fungal colonies were counted.

Assay using cellophane paper

Cellophane paper (50 mm in diameter) was placed in the centre of a Petri dish of water agar. Three *M. incognita* egg masses were placed in the centre of the cellophane paper, the controls stayed without egg masses. After 4 days of incubation at room temperature, the cellophane paper of both treatments and controls was removed and fungal plugs (7 mm) were placed in the centre of the dish in 3 replications. After 10 and 17 days, fungal colony diameters were measured.

Results and discussion

Assay using microcentrifuge tubes

All isolates showed an almost complete reduction in growth after 4 days of incubation, compared to controls without *Meloidogyne* egg masses (Figures 1 & 2). Fungal concentrations of Vcc600.88, Vc10 and Vc0083 decreased from an initial concentration of *ca* $1X10^3$ cfu/ml to $1x10^1$-$1x10^2$ cfu/ml. No cgu's could be detected in the case of *V. dahliae*. After 7 days of incubation, the concentration of all fungi decreased even more to less than $1X10^1$ cfu/ml; Vc0083 was not detectable at that day. In the control tubes, the fungal concentrations increased from *ca* $1X10^3$ cfu/ml to $1X10^4$-$1X10^5$ cfu/ml after 4 days and up to $1X10^6$ cfu/ml after 7 days of incubation. This experiment clearly shows that in the presence of *Meloidogyne* egg masses, growth of all tested fungi is reduced significantly. A difference in inhibition between the selected *V. chlamydosporium* isolates could however not be demonstrated since all the strains were inhibited with at least 99% after only 4 days of incubation.

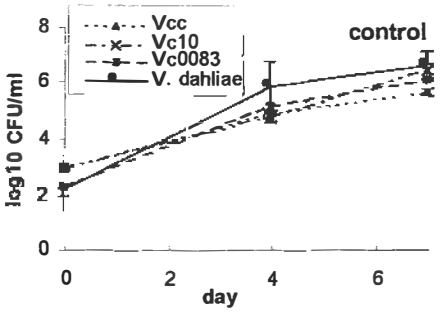

Figure 1. Growth of different *Verticillium* strains in TSB

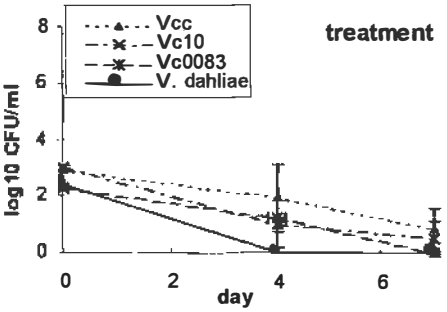

Figure 2. Growth of different *Verticillium* strains in TSB with *Meloidogyne* egg masses

Assay using cellophane paper

The *V. chlamydosporium* isolates Vcc600.88 and Vc0083 showed a growth reduction of more than 60% after 10 days of incubation on the plates that contained egg masses compared to control treatments; growth of *V. dahliae* was even more inhibited with a reduction of 70%. Vc10 showed a significantly smaller growth reduction of only 20% compared to the other isolates at day 10. After 17 days of incubation, all isolates still showed a strong growth inhibition with more than 50%, while Vc10 was only inhibited with 15%. These results show on the one hand that after 10 days of incubation the non-parasite *V. dahliae* is inhibited significantly more than the *V. chlamydosporium* isolates, as also demonstrated in the first experiment where *V. dahliae* could not be detected anymore after 4 days. (Figures 3-6). This might be due to the fact that *V. dahliae* has never been shown to parasitize nematode eggs where the *V. chlamydosporium* isolates had proven to parasitize eggs in previous experiments in our lab (data not published). These latter strains might be more resistant to the antibiotic substances and antifungal compounds present in the gelatinous matrix, most likely produced by the microbial community of the matrix. On the other hand, the fact that Vc10 was less inhibited by the egg masses might prove that strains which are originally isolated from *Meloidogyne* egg masses, are more adapted to the microbial niche of the matrix resulting in a higher resistance to the produced compounds. If this is true for all those *V. chlamydosporium* isolates, the resistance of an isolate to the gelatinous matrix could be the deciding factor for selecting promising strains. However, further research including more isolates has to be performed.

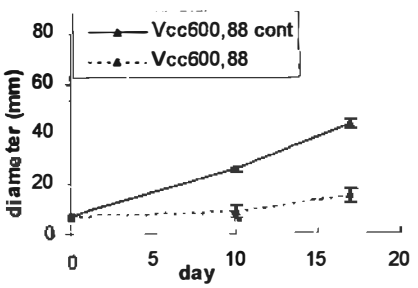

Figure 3: Inhibition of Vcc600.88 by *Meloidogyne* egg masses in cellophane assay. Vcc600.88 cont: control; Vcc600.88: treatment with egg masses

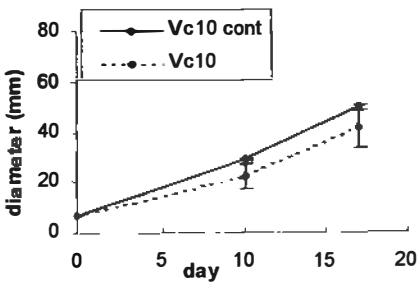

Figure 4: Inhibition of Vc10 by *Meloidogyne* egg masses in cellophane assay. Vc10 cont: control; Vc10: treatment with egg masses

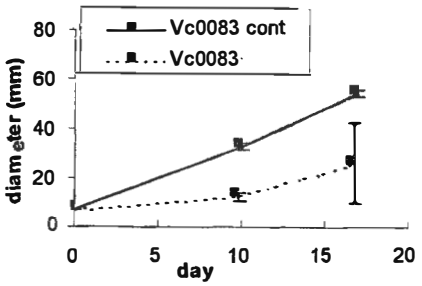

Figure 5: Inhibition of Vc0083 by *Meloidogyne* egg masses in cellophane assay. Vc0083 cont: control; Vc0083 treatment with egg masses

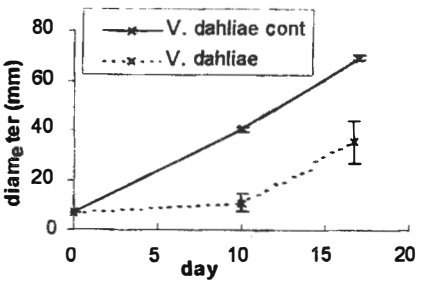

Figure 6: Inhibition of *V. dahliae* by *Meloidogyne* egg masses in cellophane assay. *V. dahliae* cont: control; *V. dahliae*: treatment with egg masses

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Crosstalk in the rhizosphere: Two *Pseudomonas fluorescens* biocontrol strains influence each other in the production of antifungal compounds

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The root-colonizing bacterium *Pseudomonas fluorescens* CHA0 protects various crop plants against diseases caused by soil-borne fungi. Strain CHA0 excretes the antifungal metabolites 2,4-diacetylphloroglucinol (Phl), and pyoluteorin (Plt) as well as the metal chelator salicylate (Sal) which are important factors in biocontrol. We have found that production of Phl and Plt and expression of the corresponding biosynthetic genes in this pseudomonad can be strongly affected by exogenous biotic signals, in particular by certain phenolic bacterial and plant metabolites. Phl biosynthesis in strain CHA0 is regulated by a positive feedback loop. The bacterial metabolites Plt and Sal, however, strongly repress Phl biosynthesis. In the same vein, Plt synthesis is strongly repressed by exogenous Phl and Sal, pointing to a mechanism which helps the bacterium to control the balance of antifungal compounds produced. *P. fluorescens* Q2-87 is another biocontrol strain which effectively controls soilborne diseases. This strain also produces Phl as a key factor in disease suppression. We were interested to test whether Phl production by Q2-87 influences the expression of Phl- and Plt biosynthetic genes in CHA0 when combining the two strains. In dual liquid cultures where the two strains were physically seperated by a membrane we found that Phl production by Q2-87 was responsible for an induction of phlA gene expression in CHA0 and on the other hand for a repression of *PltA* gene expression. The same effects could be observed in the rhizosphere of wheat inoculated with a combination of these two strains. Furthermore Phl produced by CHA0 had a stimulating effect on the expression of a Phl biosynthetic gene in Q2-87 in vitro as well as in the wheat rhizosphere. We speculate that phenolic metabolites released by bacteria and plant roots in the rhizosphere may function as novel signals modulating biocontrol efficacy in *P. fluorescens*.

Biocontrol and the soil ecosystem

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Harmful effects of pesticides on environment with the examples from Turkey

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Abstract: Chemical control applies for protecting crops from harmful organisms. The advantage of this measure among other choices is the high rate of effectiveness and quick results. But, intensive pesticide usage can cause different environmental and human health problems. In this paper harmful effects of pesticides to the environment will be discussed with some examples from Turkey.

Key words: pesticides, environment, pesticide consumption

Introduction

Chemical control that provides protection of crops from pathogens, pests, and other harmful organisms is generally the most effective procedure among the crop protection measures available. The advantage of this control measure among other choices for managing plant diseases and pests is the high rate of effectiveness and quick results. Therefore, it is widely and intensively used throughout the world. If evaluated from 1983 to 1993, pesticide usage expressed as cost generally increased. While this increase was 34% (3.4% yearly) from 1983 to 1993, it was 37% (18.5% yearly) from 1993 to 1995. But this intensive pesticide usage can cause different environmental and human health problems. Crops and animals may be exposed to pesticides due to environmental contamination of water, air and soil (Lorbeer et al., 2001). In this paper harmful effects of pesticides to the environment are discussed with some examples from Turkey.

Material and methods

For discussing harmful effects of pesticides to the environment, in this study, pesticide consumption and some characteristics of the used pesticides in Turkey were examined. In those examinations, pesticide consumption digits of Turkey were estimated from the Turkish Ministry of Agriculture and Rural Affairs data. All the pesticide consumption values were given as active ingredient (a.i). Possible harmful effects of the used pesticides to the environment were exposed according to the literature findings.

Results and discussion

Pesticide usage in Turkey

While total amount of pesticide usage in Turkey was about 8,396 tons in 1979; in 2000 the usage had increased to 12,458 tons. According to these values, pesticide consumption of Turkey increased 48.38% between 1979 and 2000. Annual increase was 2.3%. On the other hand, it can be said that pesticide consumption of Turkey is still rather low among the

European countries. While according to Oskam et al. (1997) pesticide consumption in European Union countries varies from 1.2-13.8 kg/ha, pesticide consumption in Turkey varies between 0.5-0.6 kg/ha. But, there is a very heterogenic pesticide usage in Turkey. About 2/3rd of the pesticides were used in the Mediterranean and Aegean regions of Turkey, where agriculture is very intensive (Delen et al., 2001).

Some properties of the used pesticides

Most important characteristics of the pesticides is their toxicities to human beings.In Table 1, the usage of the pesticides in Turkey whith highest oral acute toxicity is shown.

Table 1. Consumption of some pesticides whith high acute oral toxicity

		Consumption (kg or L)	
Pesticide	Oral LD_{50} Value (mg/kg)	1996	2000
Aldicarb	0.9	6,994	1,039
Azinphos-Methyl	5	26,279	36,766
Bendiocarb	34	10,708	0
Cadusafos	37	2,656	10,406
Carbofuran	8	4,608	11,288
Dichlorvos (DDVP)	25	275,380	275,524
Dinitrocresol (DNOC)	20	106,836	119,012
Endosulfan	18	275,766	166,249
Fenamiphos	8	29,275	45,203
Methamidophos	13	478,890	464,671
Methiocarb	15	14,484	61,521
Methomyl	17	10,792	9,397
Methyl-Parathion	9	294,918	349,468
Monocrotophos	8	120,252	66,474
Phosphamidon	15	17,706	5,307
Total		1,675,544	1,622,325
Ratio in All Pesticide Consumption		12.14%	13.03%

As summarized in the Table 1, the total usage of the highest oral toxic pesticides decreased in 2000, compared to 1996. In spite of the increase in the ratio of the high toxic chemicals in the total consumption, the usage of some dangerous chemicals decreased dramatically.

Some pesticides registered in Turkey have air contaminating risk (McEven & Stephenson, 1979; Roberts et al., 1999; Seiber & Woodrow, 1995; Weber, 1994). Consumption of these chemicals in Turkey are given in Table 2. According to Table 2, besides the total usage, the pesticides which have air contaminating risk in all pesticide consumption were not changed significantly from 1996 to 2000.

Table 2. The usage of some pesticides whith air contaminating risk in Turkey

Pesticide	Vapor Pressure (mm Hg)	Halflife (day)	Consumption (lt or kg) 1996	2000
Aldicarb	$200x10^{-6}$	30	6,994	1,039
2,4-D[x]	$8x10^{-6}$	10	1,937,141	1,417,764
Dichloropropene	27.7	2	158,800	424,080
Dinitrocresol(DNOC)	$105x10^{-6}$	20	106,836	199,012
Malathion[x]	$20x10^{-6}$	2	136,031	31,460
Methamidophos	$800x10^{-6}$	6	478,890	464,671
Methyl-Bromide	1702	20	779,073	322,969
MethylIsothiocyanate[xx]	20	10	85,390[xxx]	124,574[xxx]
Methyl Parathion[x]	$20x10^{-6}$	5	249,918	349,468
Molinate[x]	$5595x10^{-6}$	190	223,806	206,069
Monocrotophos	$160x10^{-6}$	30	120,252	66,474
Thiobencarb	$3x10^{-6}$	21	24,498	629
Trifluralin[x]	$103x10^{-6}$	60	758,879	890,785
Total			5,066,508	4,498,994
Ratio in All Pesticide Consumption			36.72%	36.14%

[x] Found in the air in the USA
[xx] Degradation product of *metham* sodium and *dazomet*
[xxx] Consumption of *metham* sodium and *dazomet*

The pesticides which have high solubility and volatility have risk of contamination to ground waters (Coats, 1991; McEwen & Stephenson, 1979; Somasundaram & Coats, 1991; Weber, 1994). The ratio of the pesticides which have contaminating risk to ground water, decreased in 2000 compared to 1996, from 17.71% to 10.45% respectively.

After this information, it can be said that some pesticides can have harmful effects to the environment as a result of contamination of their residues to air, ground waters and also to human health even under the low pesticide consumption conditions. Therefore, pesticides should be used under control in the areas, where considerable water resources, fertile sources and settlements are.

As a result of intensive usage of pesticides in some parts of the world as well as some parts of Turkey, effectiveness of some chemicals to the useful organisms and their resistance risks are another environmental problem. For example in Turkey, some pathogens have become less sensitive to some intensively applied fungicides (Delen et al., 2000; 1996; Delen & Ozbek, 1994). As a result of this problem, the growers tend to increase pesticide dosages to be able to control the pests. This usage of overdoses bring along serious environmental problems as summarized above.

For solving these problems, resistance risks and harmful effects of the chemicals to the environment must be taken into consideration in the registration and also in practice. Moreover, Figure and especially integrated pest management studies, including environmentally friendly pesticides, should be carried out. As a result of these studies, the chemical programs must be revised.

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The heterogeneous soil environment: Are there preferential pathways for fungal spread?

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Abstract: Most studies with soil-borne pathogenic fungi have been done with little explicit characterisation of soil structure within which fungi spread and biotic interactions occur. Soil, however, constitutes a framework of surfaces formed by old root channels, cracks or biopores in combination with aggregates. Using epidemiological and soil biological techniques in controlled environments we investigated the effect of soil heterogeneity on fungal growth dynamics. We show that cracks and larger pores can act either as preferential pathways or barriers for the spread of fungal plant pathogens through soil. Understanding the effect of soil structure on pathogen and antagonist dynamics is therefore critical for our understanding of epidemics and the development of control strategies in a heterogeneous environment.

Introduction

The structure of soil mediates all soil processes at a wide range of scales, and has significant effects on plant development, movement of water and particles, soil usage and on processes involving soil bacteria and fungi. Manipulation of soil structure is one of the principal means by which biological processes can be altered at scales ranging from the rhizosphere to the field scale (Young & Ritz, 2000). Many examples can be found where a change in tillage practice (e.g. reduced versus conventional tillage) had a significant impact on disease severity.

Field soil is not a uniform, homogeneous medium, but exhibits spatial heterogeneity on many scales. In tilled soil it can manifest itself in the form of beds of aggregates; in non-tilled soil, it may appear as a system of planar pores or cracks between soil peds. Biopores formed by roots of previous crops or macrofauna also are important aspects of soil structure. Foraging behaviour of soil micro-organisms therefore involves exploring a tortuous trajectory through a heterogeneous framework of cracks, biopores and aggregates. In spite of this the majority of studies involving soil organisms have been made in essentially homogeneous and structureless media. Such conditions favour repeatability but they conceal the heterogeneity in a natural soil. In their response to soil heterogeneity, fungi may differ considerably from bacteria as the hyphal network enables translocation of water and nutrients and exploration of pore spaces in a highly efficient way. Recent work on the soil-borne fungus *Rhizoctonia solani* however has shown that the spatial and temporal dynamics of fungal colonies are limited by the network of suitable air-filled pores (Otten et al., 1999). A particularly striking feature of this work is that a small change in the air-filled pore space makes *R. solani* switch from a small dense colony to a larger faster expanding colony. The fungus also showed a

preference to grow faster along surfaces than through soil (Otten & Gilligan, 1998). Questions need to be asked now as to what extent structured soil provides a framework of surfaces formed by root channels, aggregate surfaces and cracks, and what the consequences are for fungal spread and biotic interactions. In this paper we use *Rhizoctonia solani* as a target organism and combine epidemiological and soil biological techniques that enable quantification of fungal growth dynamics in soil to test if belowground surfaces can act as highways for fungal pathogens.

Materials and methods

Colonisation efficiency

The complicated dynamics of mycelial growth through soil are summarized by the colonisation efficiency (Bailey et al., 2000). For spread initiated from a localized substrate, the colonisation efficiency is given by the probability of successful colonisation of a particulate unit of substrate with distance from the source. Here we use the colonisation efficiency to assess the effect of cracks on fungal growth dynamics. In up to 60 replicated microcosms we introduced cracks in between the source of inoculum and the target particulate unit of substrate placed at 30 mm distance. We quantified the effect of the width, the orientation and the location of a crack for a fungus spreading over a surface and colonising a distant target. In addition, we used the colonisation efficiency to summarize the fungal growth dynamics of *R. solani* spreading *through* replicated microcosms in the presence or absence of introduced larger pores. In short, 10 units of inoculum of *R. solani* were buried at predetermined distances below the surface in replicated microcosms. The surface was observed daily for outgrowth. The distances through which *R. solani* had to spread to reach the surface were 20, 30, 40, 45, 50 and 60 mm, with 30 replicated samples for each distance. In half of the replicates, 4 larger pores (1 mm wide) were vertically introduced from the surface down to the layer of inoculum. Fungal spread was quantified as the number of replicates in which the fungus had spread through the layer onto the surface over time.

Cracks in soil and hyphal density distribution

A preliminary study was undertaken to test if a soil thin sectioning technique could be used to reveal hyphal distributions within bulk-soil and in proximity of a crack. Soil cores were prepared by packing sieved and wetted soil in polypropylene cylinders at a bulk-density of 1.2 Mg m^{-3}. Each ring contained a layer of inoculum comprising 15 g soil mixed with colonised poppyseeds (0.05 g/g) from which fungal growth was initiated. A vertical crack was introduced and the rings were subsequently incubated for 5 days at 23 °C. Thin sections were taken from horizontal layers through the soil cores (Harris et al., 2002). Sections were taken at 20, 25 and 30 mm distance above the inoculum layer. In these sections, the mean hyphal lengths were estimated in relation to the distance to the crack.

Results

Fungal spread depends on width, location and orientation of a crack

A narrow gap of approximately 0.5 mm wide was crossed by *R. solani* without any quantitative effect on fungal growth dynamics. Wider gaps up to 5.4 mm, were also easily crossed in all replicates, but did subsequently reduce the rate and extent of fungal spread. The reduction in spread after *R. solani* had crossed the crack is most likely caused by a smaller number of hyphae capable of crossing the crack with increasing crack width. The efficiency at which fungi crossed cracks depended on the location of the crack relative to the source of inoculum. The closer the crack is located to the nutrient source from which fungal growth was

initiated, the less is the effect on fungal growth. In contrast to the previous scenarios, fungal spread is significantly enhanced in the direction of the crack, with further and faster colony expansion. Considering two extreme situations with cracks either perpendicular or parallel to the direction of growth, the fungal expansion either decreased (crack acts as a barrier) or increased (crack acts as a preferential pathway).

Probability of spread through soil

The probability of *R. solani* spreading through soil to colonise a discrete nutrient target decreased with distance (Figure1). The gradual decline with distance reflects fungal morphology as the fungus becomes sparser as it grows out from the locally provided nutrient source. Larger pores increased both the extent and rate of fungal spread. We conclude that when the fungus is spreading through the bulk-soil and encounters a crack, spread in the direction of the crack is enhanced as the fungus by-passes the more tortuous pathway through the bulk-soil.

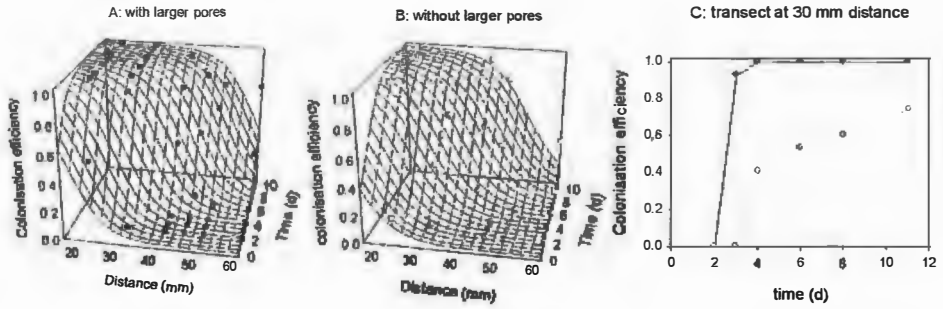

Figure 1. Colonisation efficiency of *R. solani* spreading through soil. Larger pores significantly enhance the colonisation efficiency (A) compared to samples without larger pores (B). With larger pores (C, closed circles) *R. solani* spreads faster and is more likely to colonise a target at 30 mm distance than in the absence of larger pores (C, open circles)

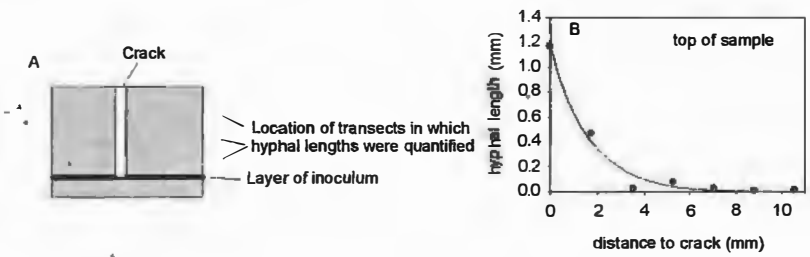

Figure 2. Schematic representation of experimental microcosms (6 cm diameter, 5 cm high) in which fungal spread was quantified (A), and an example of the hyphal density distribution in relation to the distance to the crack in a thin section taken through the top layer (B)

Hyphal densities in cracks and bulk-soil

Rhizoctonia solani was clearly visible via microscopic observation of thin sections. This enabled quantification of lengths of fungal hyphae in the crack, and in the bulk soil in relation to distance from the crack. Close to the layer of inoculum, we could not detect an effect of the crack on the hyphal density: the density of hyphae was uniform and independent of the distance to the crack. In the middle layer, at 25 mm from the inoculum layer and in particular in the top layer, at 30 mm from the inoculum, there was a higher density of fungal hyphae in areas closer to the crack (Figure 2). In the top layer, fungal hyphae were almost completely restricted to an area within 2 mm surrounding the crack. We conclude from this that fungi move initially faster and further through the crack than through the bulk soil. The precise conditions that make the fungus preferentially follow these cracks are now topic of further investigation.

Discussion

In this paper we have shown that heterogeneity in soil in the form of cracks or larger pores will have considerable impact in the spatial organisation of fungal hyphae. The effect of a crack will depend on the width, the orientation and the location of a crack and can act as a barrier or a highway for fungal spread, by-passing the soil volume. This has broad epidemiological consequences. For example it can lead to accelerating damping-off of seedlings, it will enable fungi to avoid and outrun microbial competitors, and will allow for a rapid exploitation of a crack network. For efficient control strategies it is therefore essential that we begin to understand the spatial distribution at the scale of the microorganisms.

Acknowledgements

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Research on suppression of Fusarium wilt (*F. oxysporum* f. sp. *melonis*) in muskmelon by application of bioformulations under field conditions

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Abstract: Talc-based formulations of the two strains of *P. putida* and their mixture were tested for their ability to suppress the development of Fusarium wilt, caused by *Fusarium oxysporum* f.sp.*melonis*, under in-vivo conditions. In field trials, percentage wilt decreasing effect of bioformulations, was highest on the 10th day after transplantation with values of 76%; 88%; and 84.59% for *P.putida Str.30, P. putida Str.180* and for their combination, respectively. 135 days after seeding the treated seeds into the Fusarium inoculated soil, the average values of percentage efficacy of the treatments in the plots were as follows : Str.30: 63%; Str.180: 50%; mixture: 33%.

Key words: Biofonnulation, Biological control, *Pseudomonas putida*, Muskmelon, Fusarium

Introduction

Figure of Fusarium wilts of numerous crops by application of antagonistic fungi and bacteria isolated from suppressive soils has been accomplished during recent years all over the world (Park et al., 1988; Larkin et al., 1996; Larkin & Fravel, 1998; Duijff et al., 1999; Mandeel & Baker, 1991; Alabouvette et al., 1993; Lemanceau et al., 1993; Larkın et al., 1996). In additon, several biocontrol bacteria, including *Pseudomonas* spp., *Paenibacillus* sp., and *Streptomyces* sp., have been used to control Fusarium wilt diseases (Van Peer et al., 1991; Liu et al., 1995; Raajmakers et al., 1995). Among these bacterial antagonists, different strains of *Pseudomonas putida* and *P. fluorescens* have received much attention for controlling Fusarium wilt (Weller, 1988; Liu et al., 1995; Vidhyasekaran & Muthamilan, 1995). Several mechanisms have been proposed to be involved in the suppression of *Fusarium oxysporum* by fluorescent pseudomonads (FP): (i) The suppression may be due to competition for iron (Kloepper et al., 1980), (ii) these bacteria also produce several antibiotics (Alabouvette et al., 1996), and (iii) the induced systemic resistance may be a mechanism of Figure of Fusarium wilts by FPs (Leeman et al., 1995). Application of FPs for control of soil-borne diseases depends on development of their commercial formulations in which the bacteria can survive for a considerable length of time. The objective of this research is to obtain the bioformulations of two antagonistic fluorescent Pseudomonads (*Pseudomonas putida* Str. 30 and 180) and to test their performance under the field conditions. The strains have been previously isolated and screened for their in-vitro and in-vivo efficacy on the pathosystem of *Fusarium oxysporum* f. sp. *melonis* X *Cucumis melo*.

Materials and methods

Development of bioformulations of the bacterial agents

Pseudomonas putida strains (30 and 180) found to be effective against FOM (*Fusarium oxysporum* f.sp. *melonis*) under field conditions were grown on liquid King's Medium-B for 24 hours as a shake culture incubating in a rotary shaker at 150 rpm at room temperature (24±1°C). Bacterial suspensions were then centrifuged for 20 min at 6,000 rpm. Pellets were resuspended in 0.1 M $MgSO_4$ in a ratio 1:1 (w/v). Bacterial suspensions in 0.1 M $MgSO_4$ were mixed with 10% (v/v) glycerole, which was used as a bacterial preservative. Then, this suspension was mixed with an equal volume of autoclaved 1.5 % Na-alginate (Bashan, 1986; Digat, 1990). Bacteria – Na alginate mixture was mixed with sterilized carriers such as talc, kaolinite, and whey at the ratio approximately four times the volume of the bacteria – Na alginate mixture (1:4 v/v) (Kloepper & Schroth, 1981). As a wetting agent 7% Ca-Lignine was added to the mixture. The resulting mixture was thinly spread over a glass sheet and air-dried in a laminar air flow cabine at 24 °C for 1 h to form a slightly moistened powder (15 % moisture content). After drying, bacterial formulations were powdered in a Waring Blendor and stored in glass bottles with lids as small volumes (10 g) (Suslow & Schroth, 1982; Connick, 1988; Petrolini et al., 1998).

Figure assays with the antagonist formulations under field condition

The talc-based formulations of FP strains (30 and 180) and their combination), were tested for their effectiveness to control the Fusarium wilt of muskmelon in the field trials. FOM was grown in potato dextrose liquid medium as shake culture incubating in a rotary shaker with 150 rpm at 24°C. After 4 days, cultures were filtered through cheese cloth to remove mycelial mass. Microconidial densities in the filtrate were determined by a haemocytometer and adjusted by dilution to 10^6 conidia/ml. Soil was inoculated with this inoculum. Than incubated two days. Polyethylene seedling tubes were filled with Fusarium inoculated soil. The bacterial formulations and their combination were suspended in distilled water to the end concentration of 10^9 CFU/ml. Then the muskmelon seeds were treated with the bioformulation suspensions at 50 µl per seed for 3 h and the seeds were sown in the tubes. Seeds dipped in 0.15% Benomyl served as reference. Control (+) consisted of only pathogen and the Control (-) with neither the pathogen nor the antagonist were included. The soil type was sandy loam with pH 7.2. Randomized complete block design was applied with 5 replications. The results were evaluated by using a 0-4 wilt severity scale (Sung & Huang, 1984).

Results and discussion

Fusarial wilt incidence was measured in the tubes as follows before transplanting the seedlings into field soil (Table 1). The efficacy of bioformulations during field stage is shown on Table 2. The relatively low wilt severity in the positive control plots was probably due to the high temperature pressure on the pathogen. The air temperature was measured as 30-38 °C during June, July and August 2001. Those are the first year results of the trials, the experiment will be continued next year.

Table 1. Incidence of seedlings Fusarium wilt before transplanting 33 days after seeding (%)

Treatments	Number of seedlings	Number of wilted seedlings	Percentages of wilted seedlings
Control (+)	375	34	9.06
Reference	350	5	1.42
P. putida-30	410	5	1.21
P. putida-180	371	1	0.27
30+180	480	9	1.87
Control (-)	413	0	0.00

Table 2. Values of wilt severity and percentage efficacy in the treatments, 10, 50 and 90 days after transplantation

Date of Observation	Treatments*	Ave. Wilt Severity (%)**	Average efficacy (%)
	1	3.83	76.29
25.05.2001	2	1.16	87.99
10th day after	3	2.49	84.59
transplantation	4	8.19	60.64
	5	17.80	0.00
	1	7.85	53.32
06.07.2001	2	7.21	57.13
50th day after	3	7.51	55.35
transplantation	4	10.02	40.42
	5	16.82	0.00
	1	10.76	63.21
14.08.2001	2	14.53	49.91
90th day after	3	19.47	33.49
transplantation	4	14.65	49.91
	5	29.25	0.00

*1: *P. putida* Str.30; 2: *P. putida* Str.180; 3: *P. putida* Str.30+180; 4: Ref.; 5: Control (+)

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Characteristics of *Rhizoctonia solani* isolates associated with bottom rot of lettuce

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Abstract: The AG 1-IB of *R. solani* was characterized as the predominant anastomosis group (AG) of bottom rot on lettuce. The influence of temperature on mycelial growth in vitro and on the disease development was investigated. Optimal hyphal growth was measured at 25 °C. The virulence of tested isolates varied from weak to strong. The disease incidence was also influenced by temperature. The antifungal activity of different bacteria strains was measured in dual culture. Not all strains were able to inhibit mycelial growth.

Key words: anastomosis group, biocontrol, *Lactuca sativa*, *Bacillus* sp. *Pseudomonas* sp.

Introduction

The soilborne pathogen *Rhizoctonia solani* Kühn, teleomorph *Thanatephorus cucumeris* (Frank) Donk, occurs throughout the world and has an extremely wide host range (nearly 500 plant species), especially isolates of the AG 1 and AG 2. The fungus is a collective species with 12 anastomosis groups (AGs), AG 1 to AG 12. A certain degree of host specificity may occur among AGs. *R. solani* causes bottom rot disease of lettuce (*Lactuca sativa* L.). In Germany, bottom rot is an increasing problem. Typical symptoms first appear as small rust coloured necrotic spots on leaf midribs and leaf parts in contact with soil. The causing AGs are not well documented.

In the past bottom rot on lettuce was mainly controlled by the application of fungicides. At present, neither chemicals nor resistant cultivars are available and alternative control methods are lacking. The use of microorganisms with antifungal effects against *R. solani* in practice could be a control strategy. Thus, the objectives of this study were to characterize field isolates of *R. solani* obtained from lettuce and to check the antifungal effectiveness of different bacterial strains in vitro against the field isolates.

Material and methods

During the growing seasons 1999 and 2000, *R. solani* isolates from lettuce plants with symptoms of bottom rot were randomly collected from eight commercial fields throughout Germany. The AGs of *R. solani* isolates were characterized by anastomosis behaviour with known tester isolates from AG 1 through AG 5 and morphological characteristics (Yang et al., 1989). The hyphal growth rate of 10 isolates from four locations each was measured at 10, 15, 20, 25 and 30 °C on potato dextrose agar (PDA). The colony diameter of two replications of every isolate was assessed at 24 h intervals.

The disease incidence was determined on the basis of visual symptoms after inoculation of lettuce (cv. Daguan) with *R. solani* at different temperatures (25/15, 20/10 and 15/10 °C day/night in climate chambers. Furthermore the virulence of different isolates was tested on young lettuce plants in the greenhouse. At the end of the experiment (14 days after inoculation) the dry weight of each lettuce plant was measured. In both experiments lettuce was planted in pots (500 ml) at the 2-3 leaf stage. The seedlings were inoculated with *R. solani* at the same time. Every treatment was carried out in four replications. Antifungal activity of three *Bacillus subtilis* strains (B2, E28, FZB24), one *Paenibacillus macerans* strain (E22) and one *Pseudomonas fluorescens* strain (E11) was tested against *R. solani* isolates from the four locations on PDA at 25 °C.

Results and discussion

All collected *R. solani* isolates were characterized as AG 1 and belonged to subgroup 1-IB according to sclerotial forms and types on water agar. AG 1-IB is the predominant AG causing bottom rot of field grown lettuce in Germany. Our results are in accordance with Herr (1992), who also found the AG1-IB to be the major AG causing bottom rot of lettuce in Ohio.

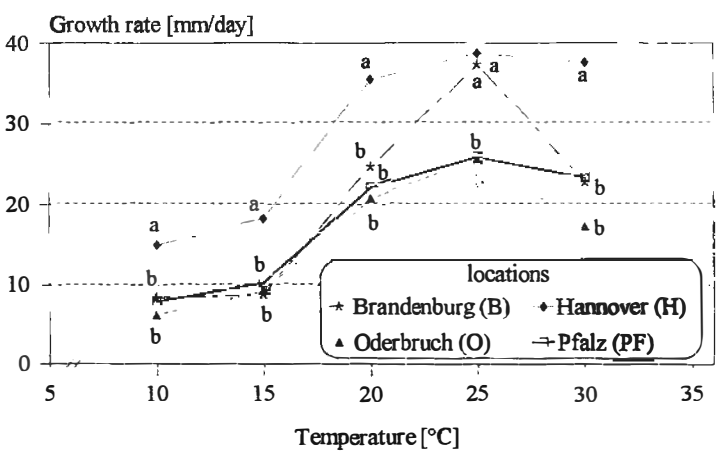

Figure 1. Hyphal growth rate of *Rhizoctonia solani* AG1-IB isolates from lettuce plants with bottom rot symptoms: average of the different locations (HSD-test, P = 0.05)

Temperatures between 20°C and 30°C favoured hyphal growth. All isolates had a more or less pronounced optimum at 25 °C (Figure 1). Statistically significant differences in hyphal growth rate were found between lettuce isolates from Hannover and isolates from the other locations at all temperatures, with one exception at 25°C with the Brandenburg isolates. The higher hyphal growth rate of the isolates collected from Hannover can be due to an adaptation to distinct environmental conditions.

The temperature influenced the disease development by *R. solani* on lettuce as well (Figure 2). The disease incidence increased with increasing mean temperature. The highest disease incidence was observed at 25 °C (day temperature). The dry weight was significantly reduced at 20 and 25 °C (day temperature) in comparison with the control. No influence on

dry weight of lettuce plants was measured at 15°C. Lettuce bottom rot caused by *R. solani* is favoured by warm, wet conditions (Davis et al. 1997). In Germany more problems with *R. solani* on lettuce exist in commercial fields in warmer regions. Thus, the results of mycelium growth and influence of temperature on disease development supported this observation.

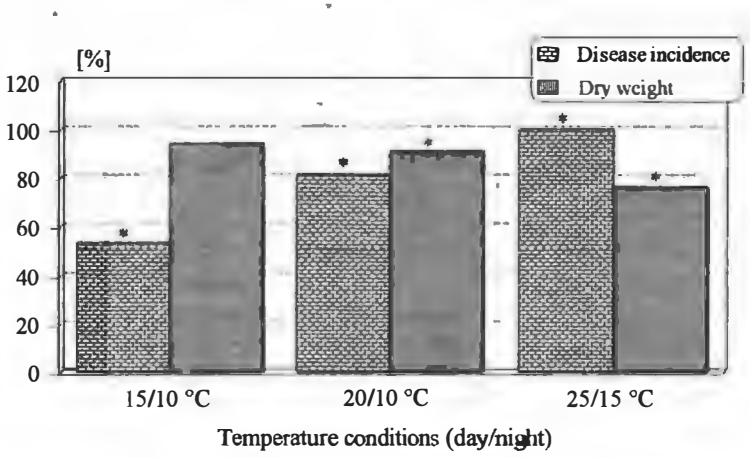

Figure 2. Percentage disease incidence and dry weight of lettuce (cv. Daguan) after inoculation with *R. solani* AG 1-IB in dependence on temperature conditions in climate chamber. (*Significant differences compared to the control, HSD-test, $P \leq 0.05$)

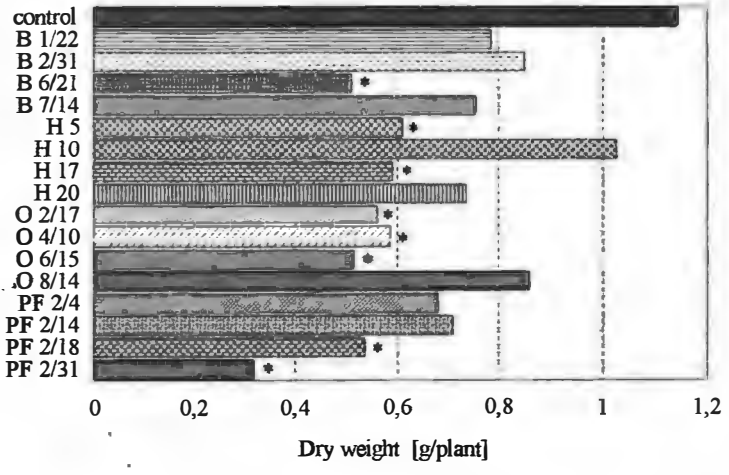

Figure 3. Dry weight of lettuce (cv. Daguan) two weeks after inoculation with *R. solani* AG 1-IB isolates from different location (*Significant differences compared to the control, HSD-test, $P \leq 0.05$)

The virulence of *R. solani* isolates in the same field varied from weak, such as isolates B 2/31, H 10, or O 8/14 to strong as for example isolates B 6/21, H 5 or O 2/17 (Figure 3). The frequency of different genotypes in a field population can influence the disease severity occurring in a crop.

Three strains of the *Bacillus* group (E 22, E 28, FZB 24) inhibited mycelial growth of all tested *R. solani* isolates, while the *P. fluorescens* (E 11) strain did not influence the mycelial growth of *R. solani* isolates (Tab. 1). The tested *R. solani* isolates differed in sensitivity against the *B. subtilis* strain B2. No relation was found between virulence of *R. solani* and sensitivity against the tested bacterial strains. The antifungal effect against *R. solani* in vivo will be tested in future experiments.

Table 1. Antifungal activity (inhibition zone) of bacteria strains (*B. subtilis* B2, E 28 and FZB 24, *P. macerans* E22 and *P. fluorescens* E11) against *R. solani* on PDA after an incubation period of two weeks at 25°C *in vitro*.

R. solani	Inhibition zone [mm]				
isolates	B2	E 11	E 22	E 28	FZB 24
B 1/22	2,5	0	12,0	9,0	11,0
B 2/31	0	0	9,0	13,5	11,0
B 6/21	0	0	13,5	13,5	12,0
B 7/14	6,0	0	11,5	9,5	11,0
H 5	0	0	14,0	14,0	13,0
H 10	0	0	11,5	10,0	11,0
H 17	6,0	0	12,5	11,0	13,5
H 20	7,0	0	12,0	14,0	7,0
O 2/17	0	0	11,0	12,0	10,5
O 4/10	5,0	0	11,5	7,5	11,0
O 6/15	0	0	12,0	12,5	10,5
O 8/14	0	0	14,0	8,0	8,0
PF 2/4	0	0	13,0	11,5	13,5
PF 2/14	5,0	0	10,5	10,0	13,0
PF 2/18	0	0	10,0	10,5	8,5
PF 2/31	0	0	10,0	10,5	13,0

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Effect of soil moisture on the survival of *Rhizoctonia solani* and *Trichoderma harzianum*

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Abstract: The effect of soil moisture on the survival of *Rhizoctonia solani* and of its antagonist *Trichoderma harzianum*, as well as on the dynamics of both fungi in the soil were studied under greenhouse conditions. Inoculum of *R. solani* was grown on rice grains, that of *T. harzianum* on wheat bran. Both fungi were inoculated at the same time, and the sowing of bean seeds to test the survival was carried out immediately after soil infestation and 20, 60, 180 and 360 days after soil infestation (DAI), and 3, 6, 12 and 18 DAI in a complementary experiment. Soil moisture was periodically monitored and kept at four levels varying from 15 to 57% (v/v). The pathogen survived in the soil and caused disease at all tested dates. However, in the first survival tests (0, 20 and 60 DAI), severity of root rot initially increased, but decreased later (180 and 360 DAI). On the other hand, dry weight of *R. solani*-infected plants was reduced in the initial tests, but increased later so that at 360 DAI values as in the treatment control were reached. Soil moisture did not affect the disease severity. The pathogen could easily be recovered even from drier soil, but in the presence of *T. harzianum* this was hardly possible. The antagonist improved the emergence of seedlings and led to higher weights of plants grown in *R. solani*-infested soil. However, when the pathogen was well established in the soil, antagonistic protection was lower. Consistent antagonistic effects were observed until 180 DAI, but at 360 DAI they were hardly detectable. The antagonist improved plant growth until 60 DAI even on plants not infected by *R. solani*. The antagonistic ability and the survival of *T. harzianum* were greater in soils held at intermediate soil moisture levels than in wet or dry soils, but were also influenced by the inoculum potential of both fungi in the soil.

Key words: epidemiology, integrated control

Introduction

Although contradictory results have been reported concerning the effects of soil moisture on the development of Rhizoctonia root rot on beans (Abawi & Pastor-Corrales, 1990; Van Bruggen et al., 1986), survival of *R. solani* in the soil seems to be favoured by low soil moisture (Ploetz & Mitchell, 1985). Conversely, survival of *Trichoderma* spp. and induction of suppressiveness to *R. solani* are apparently enhanced at moist soil (Liu & Baker, 1980).

The purpose of this study was to investigate how soil moisture can influence the survival of both *R. solani* and *T. harzianum* and the dynamics of both fungi in the soil. In addition, the development of Rhizoctonia root rot on beans and the Figure with *T. harzianum* were evaluated for a period of one year under different water regimes.

Material and methods

A long term-experiment on survival was carried out for a period of one year. A complementary short term-experiment was conducted only for 18 days focussing on the establishment of the fungi in the soil. *R. solani* (AG-4) was grown on rice grains and *T.*

harzianum on wheat bran. Soil was sieved and mixed with sand (2:1). The mixture was sterilized twice at 100°C for 24 hours. The content of each pot (800 ml of soil-sand) was poured on a tray and carefully mixed with inoculum of both fungi at 3% (w/w) in the long term-experiment and 1% (w/w) in the short term-experiment, and put back in the pot. During the experiments, the pots were weighted to monitor water loss and irrigated once a day. Soil moisture content was periodically monitored with a soil moisture sensor (*ThetaProbe,* Delta-T Devices Ltd., Cambridge, U.K.) and kept at four levels in the long term-experiment: 57, 42, 30 and 20% (v/v), and at three levels in the short term-experiment: 42, 27 and 15%. The following combinations were tested: without both fungi (rt), without *R. solani*/with *T. harzianum* (rT), with *R. solani*/without *T. harzianum* (Rt) and with both fungi (RT). To test the survival, ten seeds of bean cultivar 'Dufrix' were sown. In the long term-experiment, sowing was performed immediately after the soil infestation and 20, 60, 180 and 360 days after the soil infestation (DAI), and in the short term-experiment, immediately after the soil infestation, 3, 6, 12 and 18 DAI. After sowing, daily observations were made on plant emergence. Disease severity, plant height and dry weight were evaluated 22 days after sowing (DAS). Plants were removed and hypocotyls were evaluated to determine the disease severity according to a 1 to 9 scale adapted from Van Schoonhoven and Pastor-Corrales (1987).

Results and discussion

R. solani effectively survived in the soil in absence of host tissue at least one year after the soil infestation. However, in the first survival tests (0, 20 and 60 DAI), severity of root rot initially increased, but decreased later (180 and 360 DAI) (Figure 1). On the other hand, the relative dry weight of *R. solani*-infected plants was reduced in the initial tests, but increased later so that at 360 DAI values close to 100% were reached (Figure 2), although the population density of the pathogen at the end of the long term-experiment was relatively high (Tab. 1). *R. solani* became better established some time after the soil infestation. Disease severity and damaging effects of *R. solani* on plant development were highest in the tests at 20 and 60 DAI in the long term-experiment. Based on the analysis of all survival tests, we predicted that the maximum values of the disease severity would be reached around day 90 in the long term-experiment, while in the short term-experiment the highest disease severity was already noticed at day 34 in treatment Rt. This discrepancy is probably caused by the differences in the temperature regimes (cooler in the long term-experiment) and in the inoculum levels (higher in the long term-experiment).

Although the disease severity was consistently high on plants infected with *R. solani* also in the presence of *T. harzianum*, the antagonist frequently promoted emergence and growth of plants in the treatment RT. The population density of the pathogen recovered from soil was lower in the presence of the antagonist, while the final population density of *T. harzianum* was higher in the presence of *R. solani*. A comparison of the densities of both fungi over time in the short term-experiment shows that after the establishment phase an increase in density of *T. harzianum* propagules was accompanied by a decrease in density of *R. solani*.

In the survival tests at 20 and 60 DAI, no antagonistic effects of *T. harzianum* were detected. The high *R. solani* inoculum level used in the long term-experiment apparently increased the establishment of the pathogen and then the antagonistic protection failed, although the inoculum level of the antagonist was also high. After 6 months, antagonistic effects could again be observed, apparently because the ability of *R. solani* to survive declined. Although *T. harzianum* was isolated from soil and disease severity was lower in the presence of the antagonist at the end of the long term-experiment, the promotion of emergence and of the growth of *R. solani*-infected plants was slight in the last test at 360

DAI. Exhaustion of the suitable energy base provided by wheat bran may account for this observation (Papavizas, 1982).

Different soil moisture levels did not substantially affect the disease severity in the treatment Rt. However, a deleterious effect of wet soil on the survival of *R. solani* and *T. harzianum* was observed. The pathogen could more easily be recovered from drier soil at the end of the experiments. *T. harzianum* survived better in soils held at intermediate moisture levels than in wet or drier soils. Root rot was consistently less severe at the soil moisture levels 42 and 30% in the treatment RT (Figure 1). In the same way, the curves of the relative dry weight over time indicated that the antagonistic effects were more pronounced at the intermediate moisture levels (Figure 2). *T. harzianum* population density was highest at 42% soil moisture at the end of both experiments. Although the survival of *T. harzianum* seems to be increased at moist conditions, the activities and growth of the antagonist were not negligible at the low soil moisture content of 20 or 15%.

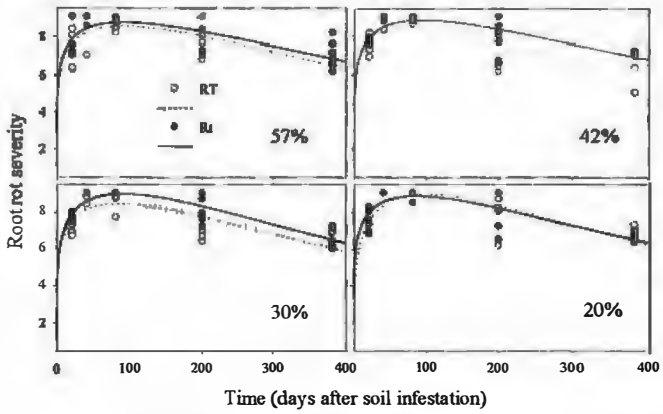

Figure 1. Root rot severity in five survival tests in the treatments RT and Rt at four soil moisture levels. The disease severities are assigned to the evaluation day at 22 DAS.

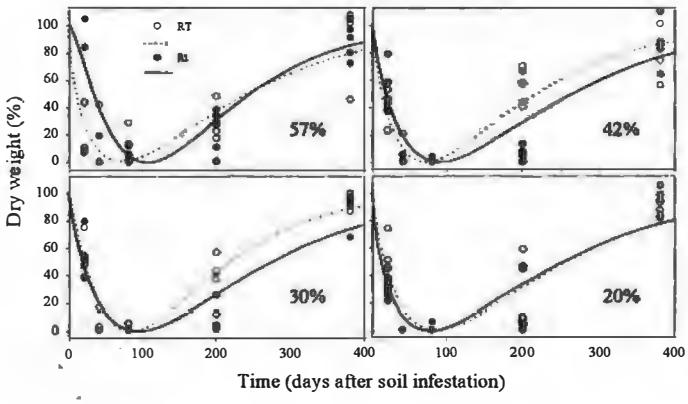

Figure 2. Relative plant dry weight (expressed as percentage in relation to the treatments rt and rT, respectively) in five survival tests in the treatments RT and Rt at four soil moisture levels. The values are assigned to the evaluation day at 22 DAS.

The antagonist improved emergence and weight of plants non-inoculated with *R. solani*, but this effect did not persist more than two months after the soil infestation. In the short term-experiment, improvement of emergence and plant weight by *T. harzianum* in treatment rT was not as expressive as in the long term-experiment. As the short term-experiment was carried out at higher temperatures than the long term-experiment, seedlings in treatment rt emerged so fast as seedlings in treatment rT in that experiment.

Table 1. Population density of *R. solani* and *T. harzianum*, expressed as cfu/g of soil, determined at the end of the long term-experiment (360 DAI).

Treatments	Soil moisture content (%, v/v)	Population density of *R. solani* (cfu/g of soil)	Population density of *T. harzianum* (cfu/g of soil)
rT	57	-	3.60×10^5 a
	42	-	4.46×10^5 a
	30	-	3.82×10^5 a
	20	-	1.28×10^5 b
RT	57	1.50 c*	6.00×10^5 a
	42	2.37 b	10.40×10^5 a
	30	2.13 bc	9.57×10^5 a
	20	3.88 a	8.80×10^5 a
Rt	57	1.70 c	-
	42	3.26 b	-
	30	3.22 b	-
	20	3.88 a	-

*Values are means of 10 replicates for *R. solani* and 5 replicates for *T. harzianum*. For each fungi combination, means followed by the same letter are not significantly different ($P \leq 0.05$).

Considering that *R. solani* persists for the longest periods in soils with low matric potential, these observations suggest that induction of suppressiveness to *R. solani* may be enhanced by manipulation of the frequency of irrigation in order to maintain the soil moist. The results presented here are of importance in the elaboration of practical Figure strategies of Rhizoctonia root rot on beans, involving water management. However, *R. solani*, *T. harzianum* and bean plants may react differently to alternating soil moisture levels compared to constant levels. Therefore, further research is needed to extrapolate our findings to the field under the wide range of moisture conditions.

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Growth relation between *Rhizoctonia* spp. isolates and soil *Trichoderma* and *Gliocladium* isolates

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Abstract: Suppression of *Rhizoctonia* spp. growth by isolates of *Trichoderma* and *Gliocladium* depended on the pathogen isolate. The pathogen population structure could have been of importance for the protective effect of the potential BCA in a forest nursery.

Key words: *Trichoderma*, *Gliocladium*, *Rhizoctonia*, suppression

Introduction

Among factors influencing biocontrol agents the pathogen itself plays an important role. The pathogen's population diversity may become a factor effecting the efficacy of natural biocontrol, also during nursery production without application of biopreparates. In Wronczyn forest nursery (Forest District Czerwonak, central-west Poland), damping-off of Scots pine (*Pinus sylvestris* L.) seedlings occurs every year, due mainly to *Rhizoctonia* spp. (Manka K. et al., 2001). In 1999 the local soil fungi community supported the pathogen growth, except for *Trichoderma* spp. and *Gliocladium viride*.

The aim of the work was determination of *Trichoderma* spp. and *Gliocladium viride* (potential biolocontrol agents; BCA) suppressivity to various isolates of the pathogen.

Materials and methods

Isolation of saprotrophic soil fungi community and Scots pine damping-off pathogens from forest nursery Wronczyn was performed at the beginning of June, 1999. Soil conditions and rotation in the nursery, sampling soil and seedlings, and isolation methods are described by K. Manka et al. (2001). Fungal community isolated from the nursery bed soil consisted of 197 isolates, of which 157 (representing 15 most frequent species = 79,8% of isolates) were taken into consideration in the biotic test.

Rhizoctonia spp. isolates obtained from the diseased seedlings were examined for the number of nuclei in cell according to Bandoni (1979) and Mikolajska & Wachowska (1996). Seven isolates of *R. solani* Kuhn (R.s. 401, 407, 410, 411, 416 and 417) and the only isolate of *Rhizoctonia* sp. (R. sp. 406) were used for the work. Three *Trichoderma* isolates (*T. harzianum*, *T. viride* and *Trichoderma* sp.) and one *Gliocladium viride* isolate represented the only BCAs present in the soil fungi community. These BCAs (in total 40 isolates) have built up 25% of the saprotrophic soil fungi community in the biotic test.

The biotic relations between the pathogens and the BCAs from the community were examined according to the biotic series method by K. Manka (1974) and Manka M. and Manka K. (1995). In an *in vitro* test the individual biotic effect (IBE) is evaluated which is the effect of one isolate of a soil fungus species on the pathogen growth. For IBE evaluation following elements are taken into account: surrounding of one colony by the other, inhibition

zone, colony growth reduction and mycoparasitism. IBE can be positive (indicating suppressive effect on the pathogen's growth), negative (indicating supporting effect on the pathogen's growth) or neutral ("0"). Intensity of the supporting or suppressing effect is described by the absolute value of the effect. The IBE is multiplied by the species frequency in the community, which results in general biotic effect (GBE = the effect of all the isolates of the species on the pathogen's growth).

Results and discussion

All the GBEs are summarized to give summary biotic effect (SBE), i.e. the effect of the entire soil fungi community on the pathogen. The SBE describes the phytopathological function of the community, as presented in Table 1.

Table 1. Biotic effect of soil fungi community from Wronczyn forest nursery on the growth of *Rhizoctonia* sp. 406.

Species of fungi	Frequency	Biotic effect on R.sp. 406	
		IBE*	GBE**
Aspergillus clavatus Desm.	33	-5	-165
Coniothyrium fuckelii Sacc.	19	-5	-95
Trichoderma viride Pers. ex Grey	17	+9	+153
Penicillium vinaceum Gilman et Abbott	14	-5	-70
Penicillium funiculosum Thom	11	-7	-77
Trichoderma harzianum Rifai	10	+9	+90
Penicillium janthinellum Biourge	10	-5	-50
Penicillium daleae Zaleski	9	-6	-54
Trichoderma sp. 1	7	+7	+49
Penicillium janczewskii Zaleski	6	-1	-6
Gliocladium viride Matr.	6	+8	+48
Penicillium chermesinum Biourge	5	+6	+24
Fusarium oxysporum Schlecht.	4	-7	-28
Chaetomium globosum Kunze ex Steud.	3	-5	-15
Chrysosporium pannorum Link (Hughes)	3	-6	-18
	157		
Summary biotic effect		**-190**	

*IBE - individual biotic effect; **GBE - general biotic effect

The soil fungi community supported the growth of all *Rhizoctonia* spp. (Table 2, Manka K. et al., 2001 ; Manka et al., 2001a, 2001b). Three *Trichoderma* spp. and *Gliocladium viride*, building up 25% of soil fungi community, suppressed various *Rhizoctonia* isolates to different extent. From among 8 *Rhizoctonia* isolates investigated, 3 *R. solani* isolates were suppressed to considerably smaller extent. The lower suppression of *R. solani* isolates 411, 416 and 417 (Table 2) by the BCAs in question, resulted in greater support of the entire soil fungi community to the three isolates (SEB –523, -518, -596, respectively, Table 2).

Trichoderma spp. and *G. viride* suppressed the only *Rhizoctonia* sp. isolate very strongly which, together with smaller support from other soil fungi (listed by Manka et al., 2001, Table

1) yielded much smaller support of the entire community (SBE –190, Tables 1 and 2) and in consequence, its single occurrence in the nursery bed.

The investigated isolates of *Trichoderma* and *Gliocladium* were successful to various extents in suppression of the pathogens' growth, depending on the pathogen isolate. It seems that the pathogen population structure can be of importance for the protective effect of a potential BCA. In the case described, weather conditions at the beginning of June 1999, with high temperature, favoring *R. solani* growth and pathogenicity (Kwasna 1987, Kacprzak and Manka M., 2001), could have contributed to the poor control effect of the BCAs in question and severe damping-off that occurred that year.

Table 2. Biotic effects of *Trichoderma* spp. and *Gliocladium viride* on various isolates of *Rhizoctonia* spp.

Rhizoctonia isolates	*Trichoderma viride* (17)	*Trichoderma harzianum*(10)	*Trichoderma* sp. 1 (7)	*Gliocladium viride* (6)	Summary biotic effect
R. solani 401	*IBE +9 **GBE +153	IBE +8 GBE +80	IBE +7 GBE +49	IBE +6 GBE +36	-369
R. sp. 406	IBE +9 GBE +153	IBE +9 GBE +90	IBE +7 GBE +49	IBE +8 GBE +48	-190
R. solani 407	IBE +8 GBE +136	IBE +8 GBE +80	IBE +8 GBE +56	IBE +8 GBE +48	-385
R. solani 409	IBE +8 GBE +136	IBE +9 GBE +90	IBE +7 GBE +49	IBE +5 GBE +30	-393
R. solani 410	IBE +7 GBE +119	IBE +8 GBE +80	IBE +8 GBE +56	IBE +7 GBE +42	-418
R. solani 411	IBE +8 GBE +136	IBE +5 GBE +50	IBE +4 GBE +28	IBE +4 GBE +24	-523
R. solani 416	IBE +5 GBE +85	IBE +2 GBE +20	IBE +5 GBE +35	IBE 0 GBE 0	-518
R. solani 417	IBE +4 GBE +68	IBE +2 GBE +20	IBE +4 GBE +28	IBE 0 GBE 0	-596

()– in brackets: frequency of the species in the soil fungi community
*IBE - individual biotic effect
**GBE – general biotic effect

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Control of root rot fungi in tomatoes with *Trichoderma harzianum*, *Bacillus lentimorbus* and solarization under glasshouse and field conditions in Chile

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Abstract: The effect of *Bacillus lentimorbus* 629 (BL 629) and of *T. harzianum* 650 (Th 650) on *Rhizoctonia solani* was tested under glasshouse and in field conditions. The biocontrol microorganisms were previously selected on the basis of their ability to control development of the pathogen *in vitro*. They were applied alone or combined with solarization and/or with methyl bromide (MeBr) in an artificially inoculated soil with *R. solani*. The control of *R. solani* by Th 650 or by BL 629 under glasshouse conditions was better than solarization and CH_3Br, both applied alone. The control degree was increased when the biocontrol agents were incorporated to these treatments. In the field experiment, the highest production was obtained after treatment with CH_3Br + BL 629, or with CH_3Br + Th 650 or with Th 650 or CH_3Br alone. On the other hand, the best treatments were BL 629 and CH_3Br + BL 629, when percentage of 1st quality fruits was evaluated. Finally, the root rot damage caused by *P. lycopersici*, *F. oxysporum f. sp. lycopersici* and *R. solani* was statistically similar in treatments with CH_3Br or with CH_3Br + BL 629, or with CH_3Br + Th 650.

Key words: *Rhizoctonia solani*, *Trichoderma harzianum*, *Bacillus lentimorbus*, *Lycopersicon esculentum*, biocontrol

Introduction

Rhizoctonia solani is a soil-borne pathogen that affects tomato crop (Latorre, 1982). Its control is accomplished through fumigation with MeBr, which contaminates the environment and affect the ozone layer (Ristaino & Thomas, 1997). The use of *Trichoderma* spp. has proved to be useful to control different phytopathogens affecting different crops (Harman, 2000). Nevertheless, different *Trichoderma* isolates behave differentially when confronted to the same pathogen (Pérez et al., 2001). On the other hand, bacteria from the genus *Bacillus* and *Pseudomonas* have also proved to control different phytopathogens (Gasoni et al., 1998). Our objective was to establish if native fungal and bacterial isolates from the genus already mentioned, applied along or without fumigation with MeBr or solarization, can control *R. solani* and other root rot pathogens of tomato.

Material and methods

Fungal and bacterial isolates

Trichoderma harzianum 650 and *Bacillus lentimorbus* were isolated from suppressive soils, and *Rhizoctonia solani* (GA4) strain 618, from tomato plants with symptoms of disease. Fungi were cultivated in Petri dishes containing potato-dextrose-agar until pure cultures were obtained. *Bacillus lentimorbus* was cultivated in King B medium as described (Montealegre et

al., 2002). Th 650 was formulated as alginate pellets, as described by Montealegre & Larenas (1997), reaching a concentration of 360.000 cfu/g pellets). BL 629 was formulated as described by Rapauch & Kloepper (1998). *R. solani* inoculum was obtained after growing the pathogen on oat seeds inoculated with agar disks containing pure cultures of the fungus.

Chitinase, β-1-3-glucanase and protease activity secreted by Th 650 and BL 629

Supernatants from submerged cultures of Th 650 or from BL 629 (Mandels et al., 1975) cultivated in the presence of cell walls from *R. solani* were used for the analysis of chitinases, β-1,3-glucanases and proteases through native electrophoresis. Enzyme activities were visualised after electrophoresis as described by Pan et al. (1987).

Treatments

Treatments were carried out applying Th 650 and BL 629 alone or combined with solarization and/or with methyl bromide in an artificially inoculated soil with 215.000 propagules per gram soil of *R. solani* in Quillota, V Region of Chile. Treatments were done after solarization and/or methyl bromide fumigation (75.5 mL(sqm) of soil, applying: a) No additions (controls); b) Th 650 formulated as sodium alginate pellets in a dosage of 1.3 g pellets/L of soil; and c) BL to roots of tomato seedlings at the transplant moment, in a dosage of 2 ml suspension of 5×10^9 cfu ml^{-1} per seedling. After treatments, plants were grown in the soil under glasshouse conditions; damage caused by *R. solani* and dry weight of plants were evaluated. Also, another experiment was run under field conditions in order to know the effect of the same biocontrol agents on naturally infected soils under high pressure of *Pyrenochaeta lycopersici*, *F. oxysporum* f. sp. *lycopersici* and *R. solani*. Production and/or aerial height and/or fresh weight and/or dry weight and/or root rot damage (Mao et al., 1998) of tomato plants were evaluated. Results were analysed according to the Duncan test.

Results and discussion

The soil content of *R. solani* of 245,000 cfu per gram was reduced to 75,000 and to 137,500 after fumigation with methyl bromide or solarization, respectively. The effect of inoculation with Th 650 or with BL 629 on tomato plants grown in artificially inoculated soil with *R solani* is shown in Table 1. The effect of these same bioantagonists on production and tomato quality is shown in Table 2. Table 3 shows the effect of Th 650, of BL 629 and of MeBr on total damage of roots from tomato plants cultivated in soils naturally infected with *R. solani*, *P. lycopersici* and *F. oxysporum* f.sp. *lycopersici*. Th 650 secreted two chitinases, four β -1,3-glucanases and high protease activity; while BL 629 did not have the ability to secrete any of the enzyme activities already mentioned. The control of *R. solani* by Th 650 or by BL 629 under glasshouse conditions agrees with those previously obtained *in vitro*, related to the ability of both biocontrol agents to prevent development of *R. solani* (Montealegre et al., 2002; Escobar et al., 2002). The secretion of enzyme systems that degrade the cell wall of the pathogen supports the ability of Th 650 to control *R. solani*.

On the opposite, BL 629, which does not secrete these enzyme systems, could be using its ability to secrete antibiotics for this biocontrol capacity. In the field experiment, the highest production was obtained after treatment with CH_3Br + BL 629, with CH_3Br + Th 650 or with Th 650 or CH_3Br alone. On the other hand, the best treatments were BL 629 and CH_3Br + BL 629, when percentage of 1st quality fruits was evaluated. Finally, the root rot damage caused by *P. lycopersici*, *F. oxysporum* f. sp. *lycopersici* and *R. solani* was statistically similar in treatments with CH_3Br, with CH_3Br + BL 629, or with CH_3Br + Th 650. It may be concluded that in terms of yield and quality, these microorganisms could replace at least in part, the use of the fumigant.

Table 1. Effect of different treatments on tomato plants grown in artificially inoculated soils with *R. solani* under glasshouse conditions

Treatments	Aereal height (cm)	Fresh weight (g)	Dry weight (g)	Damage level[1]
Control	139.6 b	157.3 a b	28.5 c d	3,0 a b[2]
Control + Th 650	168.3 a b	185.3 a b	38.5 a b	1,6 b
Control + BL 629	171.6 a b	171.8 a b	32.2 b c d	2,0 a b
Solarized	170.3 a b	124. 3 b	25.9 d	3,6 a
Solarized + Th 650	186, .6 a	188. 6 a b	42.1 a	2,6 a b
Solarized+ BL 629	178.0 A	182. 0 a b	36.7 a b c	2,6 a b
CH_3Br	156.6 A b	128.6 b	28.6 c d	3,6 a
CH_3Br + Th 650	188.6 A	210.6 a	43.9 a	2,0 a b
CH_3Br + BL	172.6 A b	200.6 a	41.7 a	2,3 a b

[1] Mao et al, 1998; [2] Different letters mean significant differences at $P \leq 0.05$.

Table 2. Effect of Th 650, BL 629 and MeBr on tomato fruit production and quality

Treatments	Total Product kg/plant	1st quality[1] kg/plant	% of 1st quality fruits	Fresh weight kg/plant[5]
CH_3Br[4] + BL 629[3]	2.1 a	1.5 a	72.7 a	7.4 b
CH_3Br[4] + Th 650[2]	2.1 a	1.3 a	62.6 ab	9.2 ab
Th 650[2]	1.9 a	1.2 ab	65.4 ab	7.9 b
CH_3Br[4]	1.9 a	1.2 ab	67.0 ab	11.3 a
BL 629[3] + Th 650[2]	1.7 ab	1.1 ab	65.9 ab	7.8 b
BL 629[3]	1.7 ab	1.2 ab	72.7 a	6.8 b
Control	1.3 b	0.9 b	61.0 a	6.4 b

[1] Harvest up to 3rd bunch 12/6/2001. Different letters mean significant differences at $P \leq 0.05$
[2] *Trichoderma harzianum* was applied 7 days before transplanting (1 g pellet (4.2×10^6 CFU/g pellet)/L soil); [3] *Bacillus lentimorbus* application: 2 ml (at 5×10^9 cfu/ml)/seedling 10 days before transplanting; [4] applied as Metabromide 980 (98 MeBr : 2 chloropicrine) (28/6/01 - 3/7/01) (aeration until 5/7/01) in a dosage of 75.5 g/m^2 before transplanting (6/7/01); [5] mean fresh weight of plants at the end of culture.

Table 3. Effect of Th 650, of BL 629 and of CH_3Br in % of total damage of roots of tomatoes cv. 593 cropped under greenhouse conditions

Treatments	Damage level *P. lycopersici*		% of total damage on roots caused by *Rhizoctonia solani*, *F. oxysporum* f.sp. *lycopersici* and *P. Lycopersici*	
Control	3.8	a	78.4	a
Th 650[(1)]	3.7	a	71.7	ab
BL 629[(2)] + Th 650	3.2	ab	65.5	b
BL 629[(2)]	3.2	ab	60.4	bc
CH_3Br[(3)] + Th 650	2.7	bc	49.2	cd
CH_3Br[(3)] + BL 629	2.3	bc	45.1	de
CH_3Br[(3)]	2.2	c	35.7	e

Different letters mean significant differences at $P \leq 0.05$; Damage 0-5 (Campbell & Shishkoff, 1990).

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biocontrol of *Phytophthora cactorum* on apple rootstocks by antagonistic fungi

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Abstract: The use of fungi as biocontrol agents against *Phytophthora cactorum* on apple rootstocks was evaluated. For this purpose, microorganisms were isolated from rhizosphere, roots, stems and soil from apple fields affected and unaffected by the pathogen. From a total of 232 fungal isolates, 8 isolates belonging to the genus *Beauveria*, *Clonostachys*, *Chaetomium* and *Trichoderma* were selected for further testing as potential biocontrol agents. Two experiments were conducted under greenhouse conditions by using the apple rootstock MM106, whose shoots were inoculated with the pathogen and/or with the potential biocontrol agents. Significant disease control was achieved by using fungicide Fosetyl-Al and seven biological treatments, when compared to the inoculated control. Among the most efficient treatments, were *Trichoderma* sp. (Th 163) from soil, *Clonostachys rosea* (Cc 006) from rhizosphere, *Clonostachys rosea* (Cc 004) from stem, and the fungicide, with 77%, 76%, 74% and 70% protection respectively.

Keywords: *Phytophthora cactorum*, rootstocks, apple, *Clonostachys rosea*, *Trichoderma* sp, *Beauveria* sp.

Introduction

Phytopthhora cactorum is a worldwide distributed pathogen, highly destructive on fruit trees of Rosaceae family such as apple trees, cherry trees, peach trees and apricot trees (Smith, 1995). *P. cactorum* has high infection and dissemination levels through irrigation water or bad drainage, by mean of its reproductive structures like sporangia, zoospores, oospores, clamydospores and mycelium (Agrios, 1978). This fungus has been devastating in Colombian plains where apple trees are cultured. In those regions it has caused economic losses near US$ 500.000.000 to fruit producers (Blanco, 1992). Current chemical control methods of this pathogen have been inefficient in almost all cases. For this reason is necessary to find control alternatives for *P. cactorum* management, such as biocontrol by using fungi, which could help to minimize yield losses. The purpose of the present work was to evaluate native fungal isolates for control of *P. cactorum* in apple rootstocks.

Materials and methods

Potential biocontrol fungi were isolated from soil, rhizoplane, rhizosphere and stem samples of apple plants collected in orchards from three different towns in Boyacá (Colombia). From each orchard, two plants were sampled, one with lesions and the other one healthy. Potential biocontrol rhizospheric fungi isolation was made by using soil washing method described by Hennebert (1996). Soil, rhizoplane and stem fungi isolation was carried out by plate count method on Czapeck Dox agar. Thereafter isolates were cultured on acidified PDA. Potentially biocontrol fungi were selected taking into account their reports as biocontrol agents and their

ability to grow at both 4°C and 24°C.

P. cactorum strain was isolated from Anna apple trees on MM106 rootstocks, and was identified by International Mycology Institute (IMI) in United Kingdom. To determine optimal pathogen concentration to induce disease, five concentrations of *P. cactorum* (400, 600, 800, 1000 and 2000 sporangia/ml) were evaluated. One ml of each suspension was inoculated to the shoot apex from the apple rootstock MM106; inoculated sites were covered with moistened cotton wool and plastic bags, plants were placed for 48 hours in a flooded bed. Daily during 20 days cotton wool was moistened to avoid inoculation site dehydration and to obtain optimal humidity conditions. A completely randomized design was used, with one shoot per plant as experimental unit; each pathogen concentration and both treated and non treated controls were considered. Each treatment consisted of five replicates. Data were recorded by daily measuring the infected area (necroses) from the third day after inoculation (when first symptoms appeared) until twentieth day. To evaluate the biocontrol activity of isolates 1 ml of a suspension containing $1x10^7$ conidia/ml suspension of each isolate was inoculated on the shoot apex, 24 and 48 hours before pathogen inoculation, which consisted of 1 ml of 800 sporangia/ml suspension on the shoot apex. After inoculations shoots were covered with moistened cotton wool and plastic bags and plants were placed in a flooded bed. The chemical product Fosetyl-Al was applied at the commercial recommended dose as explained above. Experimental conditions and evaluation of disease symptoms were carried out as described above; 11 treatments were established: a non treated control, a pathogen control, a chemical control in the presence of the pathogen, and 8 potentially biocontrol agents treatments in the presence of the pathogen. Each treatment and controls consisted of 28 replicates.

Results and discussion

From six field samples analyzed, 322 fungal isolates were obtained, 232 of them belonging to 36 genus, from these, eight final isolates were selected for their biocontrol activity evaluation according to the criteria mentioned above, one *Beauveria* sp. isolate, three *Clonostachys rosea* isolates, one *Chaetomium* sp. and three *Trichoderma* spp. isolates (Table 1).

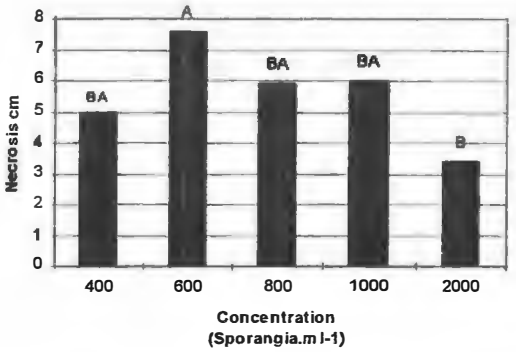
Table 1. Isolates selected for their ability to grow at 4°C and at 24°C

Genus	Isolation	Origin
Beauveria sp. (Bv 053)	Rhizosphere	Tinjacá
Clonostachys rosea (Cc 004)	Stem	Boyacá
Clonostachys rosea (Cc 005)	Rhizosphere	Tinjacá
Clonostachys rosea (Cc 006)	Rhizosphere	Paipa
Chaetomium sp. (Ch 002)	Rhizosphere	Tinjacá
Trichoderma sp. (Th 161)	Rhizosphere	Tinjacá
Trichoderma sp. (Th 163)	Soil	Paipa
Trichoderma sp. (Th 164)	Soil	Boyacá

respectively, corresponding to infection percentages of 65.7%, 77.6% and 78.9%, with non significant differences between the treatments (Figure 1). There were significant differences among 600 and 2000 sporangia/ml, being 600 sporangia/l the concentration that induced the highest shoot infection average, with 7.6 cm lesion, equivalent to 100% severity, while 2000 sporangia/ml showed a 3.4 cm lesion equivalent to 44% severity.

Figure 1. Effect of different *P. cactorum* concentrations on disease severity produced on MM106 apple rootstocks. Values with the same letter do not present meaningful differences according to Duncan's Multiple range test.

P. cactorum concentration of 800 sporangia/ml was selected because only this concentration gave disease symptoms in a steady form. When the biocontrol activity of eight isolates chosen by its ability to grow at 4°C and 24°C was evaluated, seven isolates produced



significant control, showing necrosis reduction on MM106 apple shoots compared with pathogen control. Nevertheless, for *Beauveria* sp. Bv053 treatment, disease severity was no significantly different from the pathogen control, since they showed infections of 2.55 cm and 2.92 cm respectively equivalent to infection percentages of 87.5% and 100% respectively, corresponding to 12% protection. The highest biocontrol activity was obtained with *Trichoderma* sp. Th 163, *Clonostachys rosea* Cc006 and Cc004 treatments, which showed infection averages of 0.68 cm, 0.70 cm and 0.76 cm, corresponding to 77%, 76% and 74% protection respectively. These treatments were significantly different to the obtained with *Trichoderma* sp. Th164 with a 1.33 cm lesion (54% protection), but they did not show significant differences with *Chaetomium* sp. Ch002, *C. rosea* Cc005, *Trichoderma* sp. Th161 and Fosetyl-Al treatments, that showed 0.87 cm, 1.050 cm, 1.15 cm and 1.15 cm infection lesions respectively, corresponding to 64%, 61%, 61% and 70% protection respectively (Figure 2).

lesions respectively, corresponding to 64%, 61%, 61% and 70% protection respectively (Figure 2).

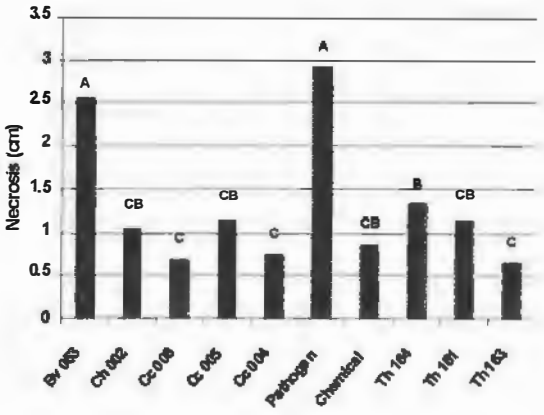


Figure 2. Effect of different potential biocontrol agents on *P. cactorum* infection on MM106 apple rootstocks. Values with the letter do not present meaningful differences according to Duncan test. Bv: *Beauveria* sp., Cc: *Chlonostachys rosea*; Ch: *Chaetomium* sp., Th: *Trichoderma* s.p.

It is important to emphasize the high protection levels showed by *Trichoderma* spp. and *Clonostachys rosea* taking into account that *P. cactorum* has a difficult control. All the evaluated fungal agents grew at low temperatures. Considering that *P. cactorum* disease development is often associated with cool, wet soil in fall and spring, these biocontrol agents are promising to control disease under natural conditions.

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Evaluation of microbial isolates for control of *Sclerotium cepivorum* in onion

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Abstract: When effectiveness of seven native microbial isolates from onions, the commercial biopesticide TRICHODEX® (*Trichoderma harzianum* T39) and the fungicide Iprodione against *Sclerotium cepivorum* was compared in a screenhouse assay. Actinomycete isolate Ac001, isolate Lv031 of the yeast *Pichia onychis* and isolate Br3129 of the bacterium *Serratia marcescens* suppressed the pathogen by 67.1%, 65.8% and 63.5% respectively and significantly better than other treatments. Trichodex suppressed *S. cepivorum* by 68.2%, but it did not present differences with these treatments with native isolates. The bacterium *Pantoea agglomerans* isolate Br3124 and Iprodione controlled the pathogen by 56.4% and 53.7% respectively, while the remaining microorganisms *Bacillus subtilis* Br006, *Bacillus lentus* Br3118 and *Paenibacillus alvei* Br3119 did so by 40.0%, 41.1% and 51.8%, respectively.

Key words: Bacteria, yeast, biocontrol agents, *Sclerotium cepivorum*, onion

Introduction

Sclerotium cepivorum is a worldwide pathogen affecting different Allium crops (Entwistle, 1990). Several control approaches have been used including chemical and physical methods (Entwistle & Coleman, 1986). Dicarboxamide fungicides, Iprodione and Vinclozolin partially protected Allium plants against *S. cepivorum* in several studies (Coley & Smith, 1987; Utkhede & Rahe, 1980; Fullerton & Steward, 1999). White rot caused by this pathogen in onion crops in Colombia has been difficult to control, resulting in major production losses, increased production costs and forced growers to move onion production to areas that are not infested by *S. cepivorum* (Ávila & López-Ávila, 1996). The purpose of the present work was to evaluate several microorganisms for control of white rot in onion.

Materials and methods

Sclerotia of *Sclerotium cepivorum* were recovered from onions in the Valley of Samacá, located in Boyacá, Colombia, surface disinfested by immersion in NaClO (2%) for 5 min, washed in sterile water for 5 min two times and incubated on potato dextrose agar medium at 15 °C for 25 days to produce more sclerotia. Potential biocontrol bacteria and a yeast were isolated from leaves, bulbs and rhizosphere of onion by dilution plating on nutrient agar medium and malt extract agar medium respectively. Biocontrol assays were performed in a screen-house using solarized soil in plastic bags (2 kg/bag) and artificially inoculated with different densities of *S. cepivorum* sclerotia (100, 300, and 500 sclerotia/kg) and with the microbial isolates. Soil in each bag was infested with 250 ml of 10^8 cells/ml suspensions of *Bacillus lentus* Br3118, *Paenibacillus alvei* Br3119, *Pantoea agglomerans* Br3124 the Actinomycete Ac001 (not

identified), *Serratia marcescens* Br3129 and *Bacillus subtilis* Br006 or with 10^7 cells/ml of the yeast *Pichia onychis*.

Two months old onion plants (cv. Yellow Granex) were immersed for 10 min in the respective microbial suspension at the same concentrations applied to the soil. One treated onion was planted in the soil in each bag 15 days after the soil was infested with the pathogen and microbial agents. A second application of microbial agents was made to the soil 40 days after transplanting using the same mentioned microbial concentrations as before.

Disease incidence was estimated at 10-day intervals for 150 days. The experimental design was a randomized complete block with three replicates per treatment. Besides microbial isolates, the biological commercial product TRICHODEX applied at 10^6 cell/kg of soil and the chemical pesticide Iprodione applied at 1.5 ml/l was evaluated. Every treatment was tested in interaction with 100, 300 and 500 sclerotia of *S. cepivorum* per kg of soil. The experiment also included a pathogen control and a control that was not inoculated and not treated.

Results and discussion

Disease incidence increased at about the same rate during 150 days in onions grown in soil inoculated with 100, 300, and 500 sclerotia/kg in the absence of the various microbial isolates (Figure 1). Highest incidence at the respective sclerotia densities were 93%, 93% and 96%. These observations are consistent with those of Entwistle (1990) who observed incidences of 69% and 94% in soil infested with 10 and 500 sclerotia kg^{-1} of soil after 150 days.

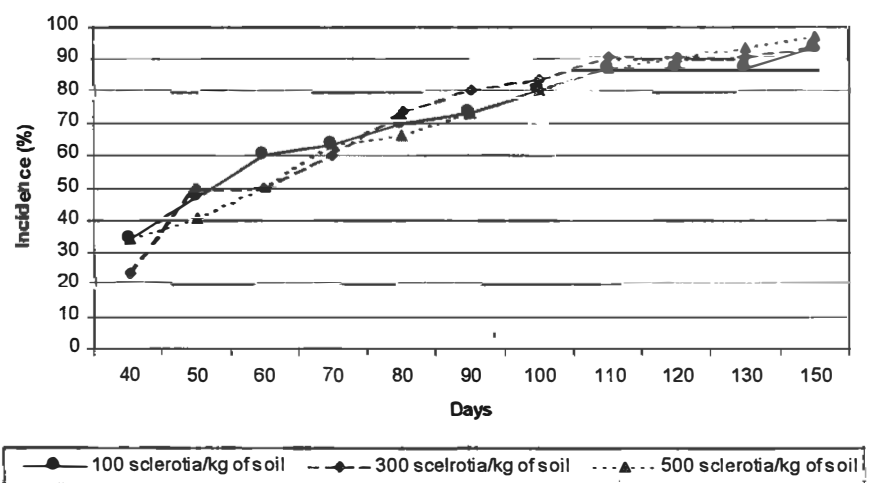

Figure 1. Progress of white rot in onions grown in soil infested with different densities of *S. cepivorum*

A total of three hundred and sixty eight isolates of bacteria were obtained from onion tissues and rhizosphere soil. Twenty eight of the isolates from different geographical areas were tentatively grouped into thirteen genera based on cultural and morphological features. One yeast also was isolated from the onion rhizosphere and identified as *Pichia onychis* Lv031. This yeast and bacterial isolates identified as *Bacillus lentus* Br3118, *Paenibacillus alvei* Br3119,

Pantoea agglomerans Br3124 and Actinomycete (Ac001, not identified) from the rhizosphere were evaluated for biocontrol activity. *Serratia marcescens* Br3129 and *Bacillus subtilis* Br006 from the Biocontrol Collection of Colombian Corporation for Agricultural Research (CORPOICA) were also evaluated.

All microbial agents exhibited biocontrol activity against the pathogen, but Actinomycete Ac001, *P. onychis* Lv031, *S. marcescens* Br3129, *P. agglomerans* Br3124 and *P. alvei* Br3119 did not present significant differences. These results were not significantly different to the results obtained with the commercial biopesticide Trichodex and the chemical product Iprodione which registered 68.2 % and 53.7% of control. The control percentage obtained with the chemical pesticide Iprodione (53.7%) coincided with results obtained by Entwistle (1990) and Fullerton and Steward (1999) who reported control percentages of 51% and 55% respectively (Figure 2). Taking this results into account we can conclude that Actinomycete Ac001, *S. marcescens* Br3129 and yeast *Pichia onychis* that presented biocontrol activities of 67.1%, 65.8% and 63.2% respectively, are efficient in controlling *S. cepivorum*.

We can suggest that microbial isolates have different degrees of competitive ability, to conquer different biological barriers, to settle down in the soil and the plant and to control the pathogen.

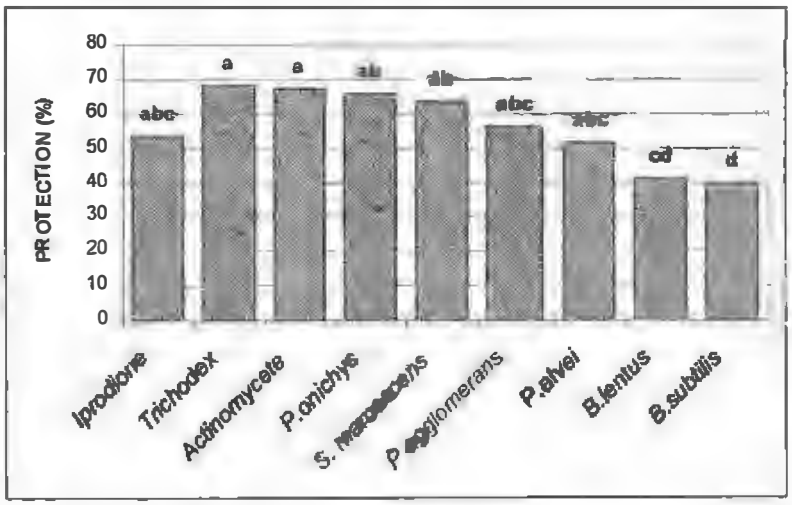

Figure 2. Biocontrol activities of different microbial isolates against *S. cepivorum* in onion under screen-house conditions. Values with the same letter do not present meaning differences according to Tukey's test.

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Prolonged shelf-life of carrier-loaded dehydration sensitive micro-organisms

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Abstract: Inclusion of biocontrol agents (BCAs) directly on seeds is an attractive supplement or even an alternative to chemical fungicides. Because the inoculum is placed were needed, direct seed application minimises the inoculum transferred to the field. Using dehydration sensitive *Pseudomonas* spp. we have shown that it is possible to apply these promising BCAs to pelleted sugar beet seeds with a sufficiently high precision and store them for more than one year without loss of biological activity.

Key words: sugar beet, *Beta vulgaris* L., seed application, carrier, shelf-life, *Pseudomonas*, rhizosphere colonisation, biological control, damping off

Introduction

Danisco Seed has for several years been investigating the applicability of biocontrol agents as a supplement to standard chemical seed treatment of pelleted sugar beet (*Beta vulgaris* L.) seeds. Focus has been put on biocontrol agents (BCAs) that are able to control the root rot causing pathogens *Pythium ultimum*, *Rhizoctonia solani* and *Aphanomyces cochlioides*. Special attention has been paid to bacterial BCAs. Some of the promising bacterial BCAs isolated from sugar beet seedlings produces the cyclic lipopeptides viscosinamide and tensin (Neiendam et al., 1998; Nielsen et al., 1999; 2000) that are believed to have antifungal and biosurfactant (detergent) properties.

Application of BCAs directly to seed minimises the inoculum need dramatically: With an efficient load of $5 \cdot 10^4$ bacteria/seed one hectar of sugar beet corresponds to a total of about $0.5\text{-}1.0 \cdot 10^{10}$ bacteria/ha, equivalent to less than 5ml of an overnight BCA culture.

One of the major obstacles in this project is to apply the dehydration sensitive micro-organisms to seeds in such a way that the micro-organisms survive the application as well as the storage on the pelleted seeds. Because of the abiotic stress put on these micro-organisms direct application will often lead to a very short shelf-life (hours). By using a special carrier preparation system even *Pseudomonas* spp. can now be applied to seeds resulting in a long shelf-life of the bacteria (> 12 months). Tests performed under controlled conditions have shown that these bacteria still have biological activity against the tested pathogens. Even after drying the carrier to very low water content necessary to avoid product contamination caused by filamentous fungi a very high survival rate can be obtained in the carrier.

Development of the special carrier system has for the first time allowed us to study the biocontrol efficiency of pellet applied dehydration sensitive *Pseudomonas* spp. in the field.

Materials and methods

Different putative bacterial BCAs (mainly *Pseudomonas* strains) were grown overnight in liquid LB-medium. The bacterial suspension was diluted with fresh LB-medium and mixed into the Danisco Seed proprietary carrier. After drying the carrier to about 20% or 5-6% respectively, the carriers were applied onto pre-pelleted sugar beet seeds by coating (approximately 60g carrier/U, 1U=100.000 seeds). All seed lots coated with BCA-carrier were without fungicides. Control seed lots were coated with carrier without BCA-load and +/- fungicides (Thiram (TMTD): 6g.a.i./U; Hymexazol: 14,7g.a.i./U). All seed lots were coated with the insecticide Imidacloprid 60g.a.i./U and covered with a final coloured cover-film.

Survival of bacteria in the original carrier as well as after application of the carrier onto sugar beet seeds was analysed after plating dilution series on solid LB medium. The number of colony forming units (CFU) per single seed was calculated as a function of storage time (shelf-life). For measuring migration of bacteria from the seed coating embedded carrier to the rhizosphere during germination, seeds were coated with carriers loaded with *P. fluorescens* strain DR54 or strain $DR54_{gfp}$, sown in peat-soil pots and germinated at 15°C or 20°C for 7 and 14 days. The seedlings were carefully harvested and most of the soil adhering to the root hairs gently removed.

Immunoblotting

Following a short surface drying the rinsed seedlings were blotted on LB-agar medium and removed. After 16 hours the blot was transferred to a nitrocellulose filter, which was then developed using specific antibodies raised against *P. fluorescens* strain DR54. For PCR the seedlings were washed in sterile water, cut into minor pieces and total DNA extracted from each of these pieces. Specific sequences in the green fluorescence protein (*gfp*) transposon cassette in strain $DR54_{gfp}$ were used as primers (Koch et al., 2001).

Field emergence trials and yield trials were done in split plot or lattice designs with 3 sugar beet varieties in 4 replications per treatment. Yield was determined as white sugar yield.

Results and discussion

The shelf-life of *P. fluorescens* strain DS96.578 coated onto sugar beet seed pellets was regularly monitored over one year (Figure 1). The shelf life of bacteria loaded into the standard carrier decreased rather rapidly to under a level sufficient for maintaining biocontrol. In contrary, by treating exactly the same carrier with the proprietary Danisco carrier treatment system (DS), the seeds now hold a sufficiently high number of culturable cells for almost one year (20% survival after 360 days).

To be of any value in a drilled-to-a-stand crop it is important that the BCAs are applied to every single seed in amounts high enough to protect the seed from the pathogens. By analysing the distribution on the single seed level it was found, that the DS carrier fulfilled these requirements (Figure 2). As can be seen from the figure, the intended average number of bacteria per single seed ($5x10^4$ CFU) was reached with a satisfactory uniform distribution.

The migration of bacteria from the seed coat to the emerging plantlet was analysed after sowing carrier-inoculated seeds in peat based soil. By using specific antibodies raised against *P. fluorescens* strain DR54 the bacteria's position could be visualised on blots. It was found that the bacteria were localised along root and root hairs, with highest intensity in the upper part of the rhizosphere. These finding were confirmed by PCR. The roots were cut into minor sections before total DNA was extracted from each of these fractions. The following PCR analysis showed that in all cases the bacterium specific sequence was detected in the upper fractions, whereas it was only detected sporadically in the root tip fractions.

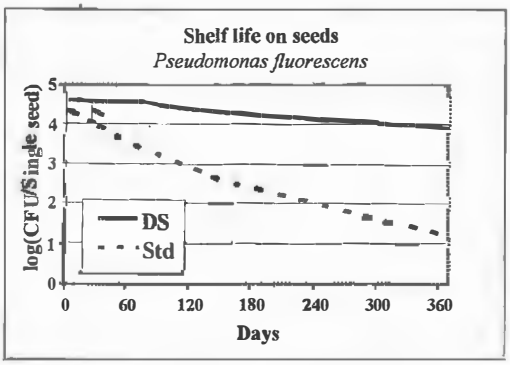

Figure 1. Shelf life of *P. fluorescens* strain DS96.578 on pelleted sugar beet seeds
Lower line: Standard carrier.
Upper line: Same carrier but treated with the DS carrier treatment system.

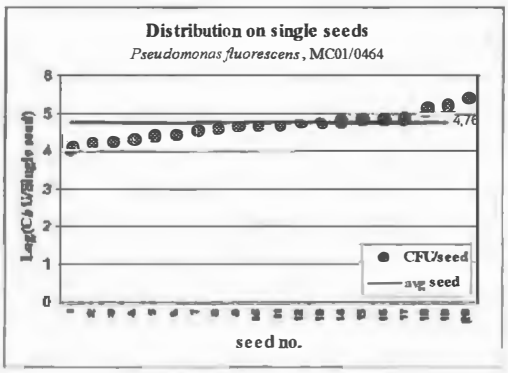

Figure 2. Distribution of *P. fluorescens* DS96.578 BCA on single seed
The BCA was applied to the sugar beet seed by coating approx. 50g of carrier per 100.000 seeds (1 Unit).

Field trials including carrier loaded BCAs were performed in year 2001. *Aphanomyces* showed to be the main cause of root rot disease in sugar beet in northern Europe. In general, seeds lots treated with Hymexazol - a fungicide active against *Aphanomyces* - resulted in higher plant establishment and higher sugar yield compared to seed lots treated with the broad spectrum fungicide Thiram, which is not as active against this pathogen. Of a total of 13 selected BCAs tested in yield trials year 2001 a few showed pathogen control at level with the Hymexazol treated seeds (See Fig 3).

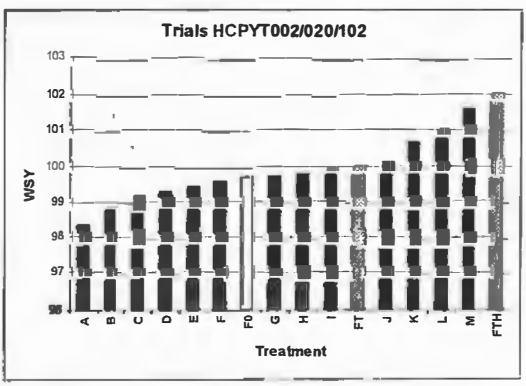

Figure 3. Yield trial 2001. Average of 3 identically designed yield trials. The result is expressed as white sugar yield (WSY) relative to the Thiram treated control (FT, 100). F0: Without fungicides. FTH: Treated with both Thiram and Hymexazol. The entries A to M are pellets without fungicides but coated with different BCAs. All seed lots were treated with Imidacloprid (60g.a.i./U) as insecticide.

Acknowledgements

We are grateful to Jan Sørensen, Tommy Harder Nielsen, Mette Neiendam Nielsen and Birgit Koch, Royal Agricultural University, Copenhagen for providing model and modified *Pseudomonas* strains and antibodies and for many fruitful discussions. We also want to acknowledge Benjamin Zilberman, UTC, Compiegne, for his study of root colonisation.

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Effective disease control on tomato and cucumber glasshouse crops by the combination of bacterial biocontrol agents

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Abstract: The aim of this work was to develop a combined biocontrol system targeting disease complexes affecting tomato and cucumber glasshouse crops in Northern Greece. *Fusarium oxysporum sp., Botrytis cinerea, Phytophthora parasitica and Pythium ultimum* were the target pathogens. The bacteria tested were, a *Bacillus* sp.(no.19), a *Pseudomonas* sp.(no.7) isolated from glasshouse soils, and *Brevibacillus brevis* (formerly *Bacillus brevis*). Antagonism by bacteria was tested alone or in combination. Combined treatments of bacterial antagonists were able to effectively control several diseases of tomato and cucumber at the same time, a new development for the control of these diseases in greenhouse crops.

Key words: *F. oxysporum sp., B. cinerea, P. parasitica, P. ultimum*, biological control agents, biocontrol, *Bacillus* sp., *Pseudomonas* sp., *Brevibacillus brevis*, tomato, cucumber

Introduction

In Pella in Northern Greece, plant pathogens are becoming an increasing problem in glasshouse tomato and cucumber due to intensive cultivation and fungicide resistance. Major pathogens include *Pythium* spp., *Phytophthora* spp. *Fusarium oxysporum, Botrytis cinerea.* Improvement of greenhouse facilities (such as heating, ventilation/ aeration) is needed for effective use of integrated biocontrol and to decrease the use of chemicals by farmers. *Brevibacillus brevis* gives moderate disease control with some of these pathogens whereas its integration with other means of disease control can lead to higher and more effective levels of control (Seddon et al., 2000). In this investigation *B. brevis* was used in combination with bacterial isolates from the crop environment in attempts to effectively control several diseases of tomato and cucumber at the same time and preliminary findings are reported here.

Materials and methods

Greenhouse soils from the Pella area cultivated with tomato or cucumber crops were selected for isolation of fungal pathogens and bacterial antagonists. Soil dilution plating for *Botrytis cinerea*, *Fusarium* spp. and bacteria and apple baiting for *Phytophthora* spp. and *Pythium* spp., was used. Classical morphological identification methods were made for fungi. Pathogenicity tests to distinguish *Fusarium* species was also used (Vacalounakis & Fragiadakis, 1999). Dual culture *in vitro* tests for antagonism of the bacterial isolates were used (Edwards & Seddon, 1992). For tomato aerial plant treatments the sowing time was 28th May 2001. On 22nd June 2001 the plants were sprayed with spore suspension of $1x10^4$/ml for *P. parasitica* and $2x10^5$/ml for *F. oxysporum* (1ml/plant), and either 1-2 day earlier or later treated plants were sprayed individually with bacteria cell suspensions (1 ml/plant) of $4x10^7$/ml for bacteria 7; $2x10^7$/ml for bacteria 19; and $2x10^6$/ml for bacteria 31.

Combination studies of aerial plant parts of tomato

Eleven trays each with four tomato plants were used with *B. cinerea* or *P. ultimum* and then with combinations of bacteria 7,19 and 31. On 28th June 2001 the plants were sprayed (1 ml/plant) with a *Botrytis* spore suspension of $2x10^5$/ml or *Pythium* of $2x10^4$/ml. 1-2 days before or 6 hr later the treated plants were sprayed with bacterial cell suspensions (1 ml/plant) as above. The control plants were sprayed with sterile distilled water. The symptoms on the treated plants with *P. parasitica* were evaluated according to a disease severity index: 1, healthy-plants free of disease symptoms; 2, low-spots on leaves; 3, moderate-plants with moderate infection on their petiole; 4, high-plants with further stem infection; and 5, dead plants. With *F. oxysporum* disease was evaluated according to a disease severity index: (H), healthy plants free of symptoms; (A), plants with 2 infected leaves; (B), plants with 3 infected leaves; (C), plants with 4 infected leaves; (D), plants with 5 infected leaves and (E), plants with 6 infected leaves; and for *Botrytis* or *Pythium* a disease severity index: Healthy, plants free of disease symptoms; Moderate, plants with 1 leaflet infected; High infected, plants with 2 leaflet or with stem or with leaf stalk infected; and Dead plants.

Cucumber combined treatments with all four pathogens, and tomato aerial plant parts treated with* B. cinerea *and combination of antagonistic bacteria

One phytotron and four seed trays with fifteen pots in each tray were used in each experiment. For cucumber and tomato the seedtime was 28th September 2001 and treatments with bacterial cell suspension (1 ml/seed each of the bacteria) of $4.2x10^8$, $4x10^8$, $2x10^7$/ml for bacteria 7, 19, 31, respectively were made. The control plants were irrigated with sterile tap water. On 7th October 2001 cucumber plants in the pathogen treated series were inoculated with fungal spore suspensions (1 ml/plant) of $5x10^6$, $1x10^6$, $2x10^5$ and $2x10^4$/ml for *Fusarium, Botrytis, Pythium,* and *Phytophthora* respectively from each fungal spore suspension. On 22nd October 2001 the tomato aerial plant parts of biocontrol treatments were sprayed with a bacterial cell suspension (1 ml/plant) as above while the control plants were sprayed with sterile distilled water. On 25th October 2001 the pathogen treated plants were sprayed with a *Botrytis* spore suspension (1 ml/plant) of $2x10^5$/ml. The symptoms on the treated plants were evaluated using the following disease severity index: 1, healthy-plants free of disease symptoms; 2, infected-plants with disease symptoms; 3, dead plants. For tomato aerial plant part treatments with *Botrytis* the symptoms were evaluated as follows: 1, healthy-plants free of disease symptoms; 2, moderate-plants with one leaflet infected; 3, high-plants with infected stem; 4, dead plants.

Results

The preliminary studies with tomato and all four pathogen (Figures 1-3) showed significant difference between treatments of (*Phytophthora*) and (P+7; P=0.034, P+19; P=0.009, 7+P, 19+P, 31+P, control, 7, 19 and 31; P=0.00) indicating activity from bacterial isolates 7, 19 and *B. brevis* (31) toward *Phytophthora parasitica.* The significant difference between (*Fusarium*) and (F+7; P=0.012, F+19; P=0.005, 7+F; P=0.002, 19+F; P=0.001, 31+F; P=0.005, control, 7, 19 and 31; P=0.00) indicated that all three antagonists seemed to reduce *F. oxysporum* colonisation (Figure 2). The significant difference between (*Botrytis*) and (B+19+31, B+7+19+31, 19+31+B and 7+19+31+B; P=0.001, control, 7, 19 and 31; P=0.00) indicated an antagonism from combinations of bacterial antagonists toward *B. cinerea* (Figure 3). The significant difference between treatment of (*Pythium*) and (7+31+P, 19+31+P and 7+19+31+P; P=0.003, control, 7, 19 and 31; P=0.001) indicated that these combinations have the ability for disease control (Figure 3).

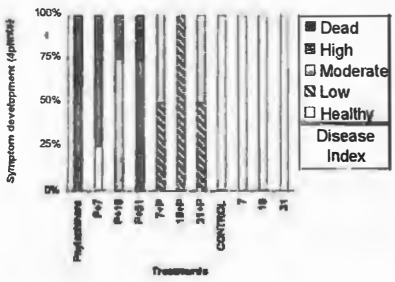

Figure 1. Disease levels at 26 days for tomato aerial plant parts treated with *Phytophthora parasitica* and bacteria

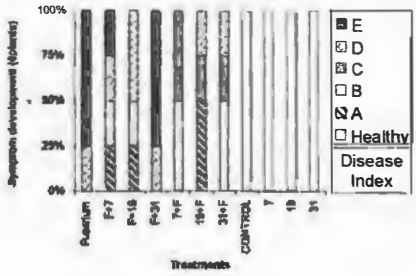

Figure 2. Disease levels at 23 days for tomato aerial plant parts treated with *Fusarium oxysporum* and bacteria

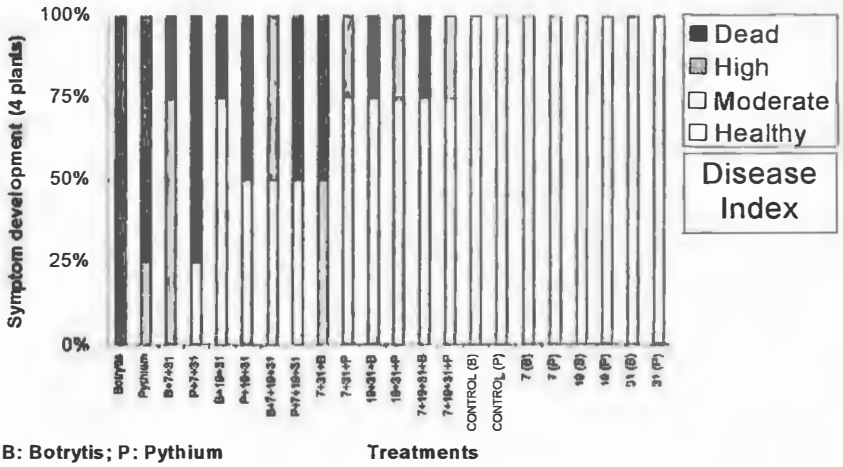

Figure 3. Disease levels at 21 days for tomato aerial plant parts treated with *Botrytis cinerea* or *Pythium ultimum* and combinations of bacterial antagonists

A highly significant difference between the treatments of (*Botrytis*) and (7+19+31+*Botrytis*, control and 7+19+31; P=0.0) at 20 days after inoculation with *Botrytis*, indicated that the combination of antagonists 7, 19 and 31 controlled *B. cinerea* to a high level (73%) (Figure 4). A highly significant difference between the treatments of (*Phytophthora* + *Pythium* + *Botrytis* + *Fusarium*) and (7+19+31 + *Phytophthora* +*Pythium* + *Botrytis* + *Fusarium*; P=0.001, control and 7+19+31; P=0.00) again indicated the ability for soil-borne disease control. Figure 5 shows that damping off, root rot, Fusarium wilt and grey mould diseases were controlled to a high level (73%) by these combined antagonists at 28 days.

Discussion

Bacterial isolate No7 (*Pseudomonas* sp.) was antagonistic to *P. ultimum* and also antagonistic activity was shown by all three bacterial antagonists (7,19,31) against *F. oxysporum* and *B. cinerea*. When this antagonism was complete these pathogens could not be re-isolated. For tomato and cucumber plants the optimal growth temperature was 14-16°C during the night

and 18-25°C during the day. *Pseudomonas* sp. (isolate 7) and *Bacillus* sp. (isolate 19) grew rapidly at 25°C. A high R.H (>90%) was extremely important for development of *B. cinerea* disease with a temperature between 16-24°C. With both crop treatments, inoculation with bacterial antagonists before pathogens gave a higher level of antagonistic activity than inoculation after the pathogen. This effect is probably due partly to establishment of a high level of antagonists and exclusion of the pathogens together with activity of antifungal metabolites (e.g gramicidin S by *B. brevis*) (Edwards & Seddon,1992). The combination of bacterial antagonists 7, 19 and 31 used in conjunction with cultural control and resistant crop plant cultivars, could prove to be an alternative approach in integrated disease control. These combined antagonists could also be used as a seed dressing at seedtime for effective disease control.

Further studies are now being made in more detail to determine the optimal parameters for integrated biological disease control.

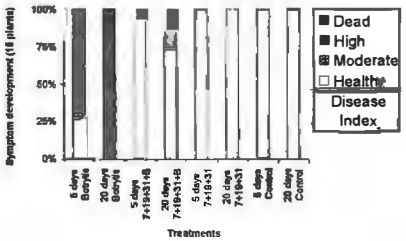

Figure 4. Tomato aerial plant parts treated with *Botrytis cinerea* and effects of 3 bacteria on disease development at 5 and 20 days

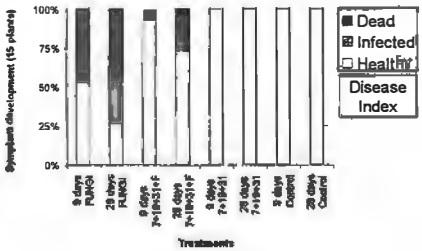

Figure 5. Combined treatments with 4 pathogens (*P. parasitica*, *P. ultimum*, *F. oxysporum* and *B .cinerea*) and effects of 3 bacteria on disease development in cucumbers at 9 and 28 days

Acknowledgements.

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Control of Fusarium wilt of tomato in soil system by combination of plant growth promoting fungus, *Fusarium equiseti*, and biodegradable pots

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Abstract: Combination of *Fusarium equiseti* and biodegradable pots (BP) were tested for ability to control Fusarium wilt of tomato caused *Fusarium oxysporum* f. sp. *lycopersici* in soil system. Protection effects of *F. equiseti* isolates GF183 and GF191 with BP based on disease severity were 50% and 67%, respectively. Development of vascular discoloration in the base of stem was also significantly suppressed by treatment of GF183 and GF191 with BP. Population level of *F. oxysporum*. f.sp. *lycopersici* in stems of tomato treated with GF183 with BP was extremely lower than that of control. Spore germination and germling length of the pathogen in the stem extract from tomato treated with GF183 with BP were significantly lower than those of control. These results suggested that suppression of pathogen multiplication in tomato plants is one of the mechanisms of disease suppression in this system.

Key words: Fusarium wilt, biocontrol, *Fusarium equiseti*, biodegradable pot

Introduction

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* causes severe damage of tomato plants grown in fields and greenhouses. As one of the methods to control this disease, resistant cultivars are used, but their effectiveness for control is not satisfactory. Another method is the usage of fungicides, however, it causes severe environmental problems. Therefore, biocontrol is an attractive alternative to control this disease. The biocontrol against Fusarium wilt has been studied intensively, but periods of experiments were short as 30 or 60 days (De Cal et al., 1995; Yamaguchi et al., 1992). We have previously shown that plant growth promoting fungus (PGPF), *Fusarium equiseti*, could control Fusarium wilt of tomato in hydroponics rock-wool system for 120 days after pathogen inoculation. The objective of this study is to control of Fusarium wilt of tomato in soil system by using combination of *F. equiseti* and biodegradable pots.

Materials and methods

Host and organisms

Tomato cv. House Momotaro was used throughout the study. Plant growth promoting fungi (PGPF), *Fusarium equiseti* isolates GF183 and GF191, were used as biocontrol agents against Fusarium wilt of tomato. *Fusarium oxysporum* f. sp. *lycopersici* (FOL) isolate Ku11 (race 2) was used as a pathogen.

Biodegradable pot

In Japan, tomato plants are grown using transplanting system. Around 1 month old seedlings grown in vinyl pots (12cm diameter) are transplanting into field soil. However, at the time of transplanting, vinyl pots were taken off and disposed as agricultural wastes which causes environmental problems. While, biodegradable pots (BP) made from grains such as corn as main raw materials could be decomposed into water and carbon dioxide through action of microorganisms once burying it in soil. So BP could be used as an alternative of vinyl pots.

Assay of combination of* F. equiseti *and biodegradable pots (BP) for control of Fusarium wilt of tomato in soil system

Paper pot set (6.5cm depth x 1.5cm diameter/pot) was filled with potting soil. Ten ml suspension of *F. equiseti* (10^7 budding-cells/ml) was drenched into each pot, and a seed of tomato which was surface-sterilized by sodium hypochlorite solution was sown in each pot. The 22-days-old seedlings were transferred to BP (9cm diameter) and drenched with 100 ml of suspension of *F. equiseti* (10^7 budding-cells/ml). Seven days later, the seedlings together with BP were transplanted into artificially pathogen-infested soil (FOL concentration was 10^4 cfu/g soil). The plants were grown in greenhouse for 90 days. Disease severity based on foliar symptom and vascular discoloration severity on the base of stem were assessed using a scale of 0 to 4 (0= healthy; 1= yellowing; 2= slightly wilting; 3= heavily wilting; 4= dead plant) and 0 to 3 (0= no vascular discoloration; 1= <1/3; 2= 1/3- 2/3; 3= >2/3 of discoloration of vascular tissue/cortex/xylem), respectively. Population of FOL in stems of tomato was estimated at the end of the experiment. The stems (1-20 cm above soil surface) were added with sterile distilled water (1:10 w/v) and homogenized using a blender at 8,000 rev min^{-1} for 8 min. The homogenized stem was filtered through two layers of cheesecloth, diluted 10 to 100 fold and plated on Komada's selective medium. The number of colony-forming units of FOL per gram fresh weight was recorded.

Effect of stem extract on the germination of pathogen

The solutions of homogenized stem of tomato from the experiment were centrifuged at 3,000 rpm for 8 min. The clear supernatant solutions were collected, and filtered in 0.45 µl Millipore. The filtrated stem extract was tested for its effect on germination of pathogen. Three ml of stem extract was added in a petri dish (40 mm diameter). Cellophane was put on the upper surface of the extract and 40 µl of spore suspension of FOL ($5x10^5$ spores/ml) was placed on the cellophane. After 6, 8, 10 and 12 hours, percent of germination was determined for 150 spores in two places per petri dish. Germ tube length for 40 spores per petri dish was measured 8 and 12 hours after placing spores on cellophane. Each experiment consisted of four replicates.

Results and discussion

In soil system, tomato plants treated with *F. equiseti* combined with BP had lower disease severity for 90 days after transplanting. Protection effects of GF183 and GF191 with BP based on disease severity were 50% and 67% at the end of the experiment, respectively (Figure 1). Development of vascular discoloration in the base of stem was also significantly suppressed by treatment of GF183 and GF191 with BP, and both protection effects were 61%. In this experiment, treatment of BP only could not control the disease (Figure 1). The reason of this might be the fast speed of BP degradation because of high temperature in soil, which could not separate plant roots from pathogen during the early stage of transplanting.

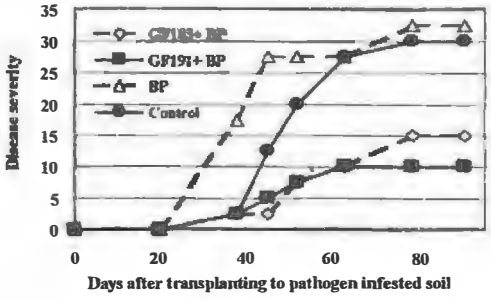

Figure 1. Progress of Fusarium wilt of tomato grown in pathogen-infested soil

Population level of FOL in stems of tomato treated with GF183 with BP was extremely lower than that of control (Table 1). This result shows that FOL could not develop and multiply well in tomato plants treated with *F. equiseti*. These results are similar with those of Nelson et al. (1992) showing that pre-inoculation of tomato and cucumber with non-pathogenic *Fusarium* reduced multiplication of isolation of pathogenic forma specialis in plants.

Table 1. Population of *Fusarium oxysporum* f.sp. *lycopersici* in stem of tomato with each vascular discoloration score

Treatment	Population of FOL ($x10^2$ cfu/g)				Average [3] Population of *FOL* ($x\ 10^2$ cfu/g)
	Discoloration score [1]				
	0	1	2	3	
GF183+ BP	0 (50)[2]	16 (30)	111 (20)	- (0)	22
Pathogen	- (0)	43 (20)	58 (80)	- (0)	56
Control	0 (100)	- (0)	- (0)	- (0)	0

1) Discoloration score; 0=no vascular discoloration, 1= <1/3, 2= 1/3- 2/3, 3= >2/3 of vascular area was discolored.

2) Percent of plants with each score.

3) Average population = Σ (population in stem with different vascular discoloration score x stem weight) / total weight.

Spore germination of the pathogen in the stem extract from tomato treated with GF183 with BP was significantly lower than that of control during 6 to 12 hours incubation of pathogen. Also germ tube length of the pathogen in the extract was significantly lower than that of control 8 hours after pathogen incubation. Suppression of pathogen multiplication in tomato plants treated with *F. equiseti* and BP is one of the mechanisms of disease suppression in this system.

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Biocontrol of phytophthora blight of pepper employing *Serratia plymuthica* A21-4 and effect of soil population of *Phytophthora capsici* on the root colonization of the antagonistic bacteria

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Abstract: A biocontrol agent, *Serratia plymuthica* A21-4 was selected from the roots of onion plant. A21-4 inhibited significantly the mycelial growth, zoosporangia formation and cystospore germination of *P. capsici in vitro*. In pot experiments, A21-4 showed 100% disease control and in greenhouse experiments, the disease control efficiency of A21-4 was 82.6%. A21-4 readily colonized on the root of pepper plant and the bacteria moved to newly growing roots continuously. More than 10^6 cfu/g root of A21-4 is needed to protect pepper roots from infection of *P. capsici* in soil. The colonization of A21-4 on the pepper root was influenced by presence of *P. capsici* in the soil. When *P capsici* was introduced in advance, the population density of A21-4 on the root of pepper plant was sustained more than 10^6 cfu/g root until 3 weeks after transplanting. On the other hand, in the absence of *P. capsici*, the population density of A21-4 was reduced continuously after transplanting and did not reach 10^5 cfu/g root, but this phenomenon did not occur in the populations of A21-4 in soil.

Key words: Pepper, Phytophthora blight, *Serratia plymuthica*A21-4, root colonization

Introduction

The damage of phytophthora blight of pepper is frequently destructive hence it is considered as principal limiting factor in pepper production in Korea. Although numerous attempts have been made to control the disease biologically, most of them were not practically feasible. Main reason is that the population density of antagonists was not sufficient to suppress the pathogen throughout the growing season. Root colonizing biocontrol agents are ideal to protect roots from pathogens in soil. In this study, we investigated a root colonizing biocontrol agent *Serratia plymuthica* A21-4 and the influence of soil populations of *P. capsici* on the colonization of A21-4 in pepper roots.

Materials and methods

Antagonistic strain and pathogen preparation

The biocontrol agent, *Serratia plymuthica* A21-4 was originally isolated from the root of onion (*Allium cepa* L.). Strain A21-4 was grown at 28°C in Tryptic Soy Agar or broth(TSB). and stored at –70°C in TSB containing 20% glycerol. The strain A21-1 was marked with rifampicin resistance for recovery. *Phytophthora capsici* Pa-61 (KACC 40476) was grown on V8 juice agar. For the production of zoosporangia, 4-5 ml of distilled water was added to *P. capsici* culture on V8 juice agar, and illuminated with blue fluorescent light at 20-25 °C, for 16 hours. The zoosporangia formed on agar surface were collected and placed at 4°C for 10-30 min then move to room temperature condition for zoospore discharge from zoosporangia. Collected zoospores were diluted to 10^4 zoospores/ml for inoculations.

Analysis of population density of A21-4 and P. capsici in soil and pepper root

One gram of root or soil samples were macerated and suspended in 9ml of 0.1M $MgSO_4$ and shaken for 1 min to take out bacteria from soil and root. The population density of A21-4 was determined by dilution plate on 1/10 strength of TSA containing 50 μl/l rifampicin. Colony forming units of *P. capsici* in soil or pepper roots were enumerated using Corn-meal agar with supplement of antibiotics (Pimaricin 0.4 bml, Rifampicin 10 mg, Ampicillin 300 mg, Hymexazole 150 mg, PCNB 300 mg per 1000 ml). One gram samples were placed on the media and incubated for 24 hours at 25°C then the plates were washed with flowing tap water and 10 ml of sterilized distilled water plus antibiotics were added. After 24 hours incubation at 25°C, excess fluid was discarded (dried 30 min) then numbers of fungal colonies were examined.

Evaluation of disease suppression

The roots of 50-days-old pepper seedlings (*Capsicum annuum* cv. Nok-Kwang) obtained from the commercial plug nursery company were dipped in the bacterial suspension (10^9cfu/ml) for 1 hour and transplanted to the greenhouse soil previously inoculated with zoospore suspension (10^4cfu/ml) of *P. capsici*. After transplanting, the infected plants showing typical phtophthora blight symptom were carefully examined. The pepper plants in greenhouse were maintained as ordinary farming practices in Jinju area.

Results and discussion

***Antifungal activity of A21-4 to* P. capsici**

Strain A21-4 inhibited significantly the germination of cystospore and zoosporangia of *P. capsici*. In sterilized distilled water the germination rate of cystospores and zoosporangia were 85% and 98% at 8 hours incubation, however that of A21-4 were only 7% and 11% respectively (Table 1). No zoosporangia were formed in plates treated with A21-4 cell suspension, but a lot of zoosporangia were formed in sterilized distilled water.

Table 1. Effect of *S. plymuthica* A21-4 on the germination of cystospores and zoosporangia of *P. capsici* on water agar.

Treatment	Germination rate (%)							
	Cystospores				Zoosporangia			
	2hr	4	6	8	2hr	4	6	8
A21-4	0	3.6	4.6	7.4	7.8	8.1	9.8	10.8
Distilled water	56.4	60.3	72.7	84.8	79.1	89.4	97.8	98.2

Inhibition of population density of* P. capsici *in soil and pepper root

The population of *P. capsici* inoculated in form of zoospore suspension was rapidly diminished in the soil without host plant, however, the fungal population in soil in the presence of host plant increased very rapidly. On the other hand, the same fungal population in soil inoculated with A21-4 was not increased rather reduced slowly. *P. capsici* immediately colonized in pepper roots and the population was increased sharply from 14 days after treatment while in A21-4 treated plant, root colonization of *P. capsici* was delayed and the increase was negligible (Figure 1).

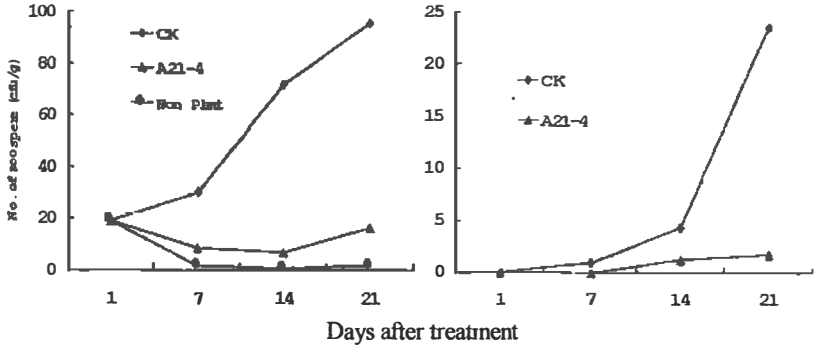

Figure 1. Inhibitory effect of *S. plymuthica* A21-4 on the increase of population densities of *P. capsici* in pepper planted soil (A) and colonizing on pepper root (B)

Colonization of A21-4 on pepper root and soil

A21-4 readily colonized on the pepper root and moved to newly emerging root continuously. The bacterial density on the root was maintained above 10^6 cfu/g root. The bacteria also moved to surrounding soil but the population density was gradually reduced (Table 2).

Table 2. Colonization of *S. plymuthica* A21-4 on the pepper root and soil after roots of pepper seedlings were soaked in the bacterial suspension for 1hr

Sampling site	Population densities after transplanting (Log cfu/g)		
	7days	14days	21days
Initial root	6.5	6.2	6.1
Newly emerging root	6.4	6.1	5.7
Surrounding soil	7.2	7.0	6.1

Influence of* P. capsici *on the colonization of A21-4

The colonization of A21-4 in the soil was not effected by presence of *P. capsici* but colonization in pepper roots was significantly influenced. The population density of A21-4 colonized on the root gradually decreased in the absence of *P. capsici* but the population in the presence of *P. capsici* did not decrease rather increased.

Table 3. Influence of pathogen *P. capsici* on the colonization of antagonist *S. plymuthica* A21-4 in pepper root and surrounding soil

Sampling site	Presence of *P. capsici*	Population Density(Log cfu/g)		
		7 days	14 days	21 days
Pepper Root	Presence	6.6	6.1	6.7
	Absence	6.9	6.2	5.6
Surrounding Soil	Presence	7.3	7.0	6.1
	Absence	7.5	7.2	6.4

Disease suppression by A21-4

When the pepper seedlings were dipped in cell suspension of A21-4 and transplanted in the greenhouse, the bacteria successfully suppressed phytophthora blight of pepper. Incidence of disease after 60 days after transplanting was 72.4% in untreated plot, on the other hand, only 12.6% of plants showed disease symptom in A21-4 treated plants (Table 4).

Table 4. Suppression of phytophthora blight of pepper in the greenhouse by *S. plymuthica* A21-4 when the roots of pepper seedlings were soaked in bacterial suspension before transplanting

Treatment	Diseased plant (%)
A21-4 Root-Dipping treatment	12.6
Untreated	72.4

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The possible systemic induction of resistance in some vegetables by fungicide resistant *Trichoderma* isolates

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Abstract: The practical application of *Trichoderma* as biocontrol agents integrated with chemical treatments required the selection of fungicide resistant isolates. We have been selected several benzymidazole and dicarboximide resistant stains of *Trichoderma viride*. Some fungicide resistant strains showed strong mycoparasitic activity and high activity of extra-cellular hydrolytic enzymes (proteases, cell wall degrading enzymes). They were also active rhizosphere and root colonisers.

The introduction of biocontrol agents into the potting compost during production of vegetable transplants, under greenhouse conditions, revealed that they produced a well-pronounced increased growth response at the early growth stages of cabbage, tomato, leek and cucumber. The dry weight of young vegetable plants grown in potting compost amended with *Trichoderma* isolates (~10^6 c.f.u. per dcm^3) was significantly higher (40 – 118%) in comparison with plants grown in untreated control compost. Among tested plants the response of tomato was most profound. The increase of dry weight of cabbage and tomato during early stage of development was correlated significantly with increase of the PAL activity in plant tissue. We found also higher PAL activity in plant tissues of other tested plants treated with biocontrol agents. The stimulating effect of tested isolates of *T. viride* was not related to the antibiotic production *in vivo*.

Under field conditions the combined application of chemical soil treatment (dazomet or 1,3-D+CP) or fungicide seedling treatment (iprodione) or drenching (tiophanate) with application of biocontrol agent, during transplant production and again during planting time, revealed significant increase of yields of vegetables (celeriac, leek, cabbage, tomato). Similar effects were observed for pepper grown in unheated plastic tunnels. The combined applications of lower rates of 1,3-D+CP (300 l per ha.) or dazomet (300 kg per ha) with biocontrol agent was superior to those chemicals used alone at higher rates, 450 l and 400 kg per ha, respectively. Under field conditions the celeriac responded to tested treatments much stronger than cabbage and tomato. Also the combined application of fungicide treatment of seedling or plant drenching with biocontrol agent was superior to fungicide chemicals or biocontrol agent used alone in production of leek under field conditions. The application of biocontrol agents under field conditions resulted with higher PAL activity in leaf extracts of most tested vegetables. However, the increase of PAL activity was significantly correlated with increase of celeriac yield, only. The observed stimulating effects of *Trichoderma viride* on the growth of vegetables suggest the induction of systemic resistance occurs and resulted in suppression of minor soil-borne pathogens.

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The influence of Cedomon® on yield and fungal infection of spring barley in field conditions in Poland

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Abstract: For testing the efficacy of Cedomon® six field experiments with 7 cultivars of spring barley (Barke, Kinnan, Rabel, Rambo, Rataj, Rudzik, Scarlet and Rudzik simultaneously sown with clover) were conducted in the years from 1998 to 2001 at three locations (north-west, south-west, and central-south) in Poland. The average increase in the yield of tested cultivars after Cedomon® treatment was found in the range from 4.3% to 10.1%. The application of *Raxil®* or *Vitavax®* as a seed dressing resulted in the average yield increase in the range from 11.7% to 17.2% and from 5.0% to 9.7%, respectively. Average efficacy of controlling of *Drechslera teres* with Cedomon® was found in the range from 28.2% to 93.8% at shooting up stage and in the range from 28.2% to 72.4% for ears formation time. The efficacy in years and locations with lower temperatures and higher precipitation in April was noticeable better than in others. The efficacy was noticeable better in the year 2001 than in pervious years. The average efficacy of controlling of *D. teres* by *Raxil®* and *Vitavax®* was found at a similar level. The average number of infected plants by soilborne fungi causing seedling damping-off on plots treated with Cedomon® or treated with fungicides were from 77.7% to 90.0% and from 66.6 to 93.4% lower, respectively. A considerable increase in the content of ARs was observed solely in seeds of Scarlett and Rabel (1999), whereas no stimulating effect was observed in straws. Nevertheless, the effect of Cedomon® was manifested by changes in homologue patterns.

Key words: biofungicide, spring barley, fungicides, integrated control

Introduction

At the end of the last decade Swedish BioAgri A.B. company elaborated the biofungicide Cedomon® which is already registered in Austria, Finland, Norway and Sweden. The active ingredient in this product is the commonly occurring soil bacterial species *Pseudomonas chlororaphis*. The bacterial strain has not been genetically modified. The bacterium *P. chlororaphis*, strain MA342, was originally isolated from *Empetrum nigrum* (Hökeberg, 1998). This strain was found to be an active biocontrol agent against a number of cereal seed-borne diseases in many field experiments with barley, oats, wheat and rye carried out at different locations in Sweden from 1991 and from 1994 in more than 10 European countries (Hökeberg et al., 1998; Johnsson et al., 1998). The bacterization controlled seed-borne diseases caused by *Drechslera (Pyrenophora) graminea, D. teres, D. avenae, Ustilago avenae, U. hordei*, and *Tilletia caries*, as effectively as guazatine + imazalil. The effects were consistent over the years and diverse climate regions. Diseases caused by pathogens like *U. nuda* were not controlled and the bacterization gave lower effect than full level against

diseases caused by *Microdochium (Fusarium) nivale*, and *Bipolaris sorokiniana (Cochliobolus sativus)* (Hökeberg et al., 1998; Johnsson et al., 1998).

The effectiveness of controlling seed-borne pathogens by strain MA342 is attributed to the production of an antifungal compound, 2,3-deepoxy-2,3-didehydrorhizoxin (Hökeberg 1998), which is probably produced in the glume of barley seeds strongly colonised by this strain (Tombolini et al., 1999). The resistance of cereals to the fungal infection of plants has been associated with the presence of non-isoprenoid phenols, so-called 5-*n*-alkyloresorcinols (ARs), which have been reported as growth-inhibitors of several fungi pathogenic for cereals (Heinzen et al., 1996; Garcia et al., 1997; Zarnowski et al., 1999). Due to their strong antibacterial and antifungal activity, those compounds are biosynthesised specifically during seedling stage to protect the plant against external aggression and predators (Suzuki and Yamaguchi, 1998). However the 5-*n*-alk(en)ylresorcinols, have been widely recognised since the 1930s as toxic and allergic constituents that may cause contact dermatitis (Anderson et al., 1931; Wasserman & Dawson, 1948). The importance of alkylresorcinols in the diet was readily demonstrated in a few reports (Pawlik, 1979; Sedlet et al., 1984).

Materials and methods

The efficacy of Cedomon® in six field experiments with 7 cultivars of spring barley (Barke, Kinnan, Rabel, Rambo, Rataj, Rudzik, Scarlet and Rudzik with simultaneously sown clover) at three locations was tested from 1998 to 2001. The fungicides *Raxil*® and *Vitavax*® were applied in 1998-2000 and 2001, respectively. The untreated seeds were used as control. The experiments were carried out; South-west of Poland at Pawłowice, Experimental Station of Agricultural University of Wrocław (1998-2001) on grey brown podzolic soil, light loam on medium-heavy loam; North-west part of Poland at Lipniki, Experimental Station of Agricultural University of Szczecin (2000) on acid brown soil, sandy clay of boulder origin; Central-south of Poland at Bakow Experimental Station of Experimental Breeding and Plant Acclimatisation Station of Smolice Co. Ltd. (2001) on podzol soil, sandy loam on medium-heavy loam. The soil fertilisation, weed and insects control were done in each location according to good agronomic practice. Each experiment was set-up as strict experiment with random blocks with 4 rps. Seedling damping-off (*Fusarium* spp., *Rhizoctonia* spp.) was evaluated after germination, net blotch of barley *(Drechslera teres)* was evaluated twice. Primary infection was determined at the beginning of shoot-up stage and the secondary infection was determined at the stage of ears formation. The observations were done on 100 plants using scale from 0 to 9 and degree of infestation was transformed according to Townsenda-Heubergera formula. The number of germinated seeds and the number of stalks formed per 1 m^2 were enumerated. The yield of harvested grain was calculated for 15% moisture. After the harvest and drying the weight of 1000 seeds, nitrogen, phosphorus and potassium concentration in grain were determined. The content of ARs was determined in seeds covering waxes by acetone extraction as was described by Żarnowski et al. (2002).

Results and discussion

The most profound effect of Cedomon® as seed treatment was observed at Pawłowice in 2000. The yield increase was found at the level of 29% compared to the untreated control. The effect of fungicide seed treatment resulted in 11% greater yield, only. The average yield increase of spring barley after Cedomon® treatment in comparison with untreated control plots in six field trials was found in the range from 4.3% to 10.1% (Figure 1). The application

of *Raxil*® or *Vitavax*® as a seed dressing resulted in an average yield increase in the range from 11.7% to 17.2% and from 5.0% to 9.7%, respectively (Table 1).

Table 1. The effect of seed treatments on the average yield, on the average net blotch index and on the average incidences of seedling damping-off of spring barley in field experiments in 1998 - 2001 at three locations in Poland

Treatments	Tested cultivars							
	Rabel	Rambo	Rataj	Scarlet	Rudzik	Rudzik + clover	Barke	Kinnan
	Yield dt per ha							
Control	3.42 a	3.33 a	3.12 a	3.66 a	2.97 a	2.83 b	4.31 a	4.97 b
Cedomon®	3.64 a	3.51 a	3.27 a	3.77 a	3.25 a	3.05 ab	4.64 a	5.00 ab
Fungicide	3.65 a	3.71 a	3.55 a	3.96 a	3.34 a	3.23 a	4.52 a	5.45 a
	Net bloch index (%)							
Control	8.57 a	8.66 a	9.42 a	8.13 a	8.30 a	10.38 a	6.54 a	19.09 a
Cedomon®	4.12 a	5.06 ab	5.00 b	3.66 b	2.18 b	3.60 b	2.27 b	4.37 b
Fungicide	5.19 a	3.90 b	5.17 b	3.47 b	1.93 b	2.13 b	4.51 b	5.78 b
	Seedling damping-off (average number of infected seedlings per plot)							
Control	3.19	3.50	4.83	4.75	3.17	5.92	2.38	3.75
Cedomon®	0.50	1.00	1.00	0.75	0.33	0.91	0.25	1.25
Fungicide	0.13	0.63	0.42	0.44	0.33	0.50	0.25	0.25

* The numbers in the columns in each subsection followed by the same letter are not significantly different according to t Student test ($P \leq 0.05$).

The estimation of plant infestation by *Drechslera teres* during vegetation showed divergence in the appearance among tested cultivars, locations and years. The primary plant infestation by this pathogen as well as a secondary infection was in most cases significantly lower on plots with chemically or biologically treated seeds. Average efficacy of controlling of *D. teres* with the biological agent Cedomon® in different years was found in the range from 28.2% to 93.8% during spreading time (Figure 1) and in the range from 28.2% to 72.4% during ears formation period. The efficacy in years and locations with lower temperatures and higher precipitation in April was noticeable better than in others. Moreover, the efficacy of biological treatment was noticeable better in 2001 (ranging from 88.0% to 95.6%) during spreading time than in pervious years probably due to improved formulation of Cedomon®. The average efficacy of controlling of *D. teres* by *Raxil*® and *Vitavax*® was found at similar level. Moreover, the average number of infected plants by soilborne fungi causing seedling damping-off on plots treated with Cedomon® and treated with fungicides ranged from 77.7% to 90.0% and from 66.6% to 93.4% lower, respectively. Tested treatments did not influence the chemical composition of harvested grain and did not effecte the mass of 1000 seeds.

The barley cultivars were also examined in 1999 and 2000 for the presence of alkylresorcinols (ARs), natural non-isoprenoids exhibiting strong fungicidal activities against various plant pathogens. A considerable increase in the content of ARs was observed solely in seeds of Scarlett and Rabel in 1999 (about 30-40%), whereas no stimulate effects were observed in straws. Nevertheless, the effect of Cedomon® was manifested by changes in homologue patterns. The considerable induction of long-chain ARs was revealed in Scarlett & Rambo (1998) as well as Rabel & Rambo (1999). Similarly, Scarlett & Rataj in 1999 contained more medium-chain ARs. The results presented here proved Cedomon® to be a

good stimulus of the alternations in ARs contents and compositions. However this process was without a shadow of doubt strongly cultivar and climate dependent.

The observed induction of alkyloresorcinols' metabolisms might results from changes in physiological status of plant organisms as an effect of the metabolic activity in barley tissues of tested biocontrol agent. In addition, the phenomenon of ARs induction (which is equivalent to the increased plant resistance) might have an additional protective effect from fungal infestation. However, the increase in the content of alkylresorcinols in grains of barley is not a threat to human and animal health.

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The interrelationship between the ability of wheat seedling colonisation and certain physiological properties of *Pseudomonas*

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Abstract: Forty rhizobacteria from the genus *Pseudomonas* were tested for their ability to colonise wheat seedlings. Upon their ability for multiplication on seedlings they were classified into three groups: very good colonisers, good colonisers, weak colonisers. The ability of these bacteria for seedling colonisation was partly correlated with the ability for utilisation of D-galactouronic acid, D-glucouronic acid, ferulic acid and *p*-coumaric acid, adonitol, *p*-hydroxyphenylacetic acid, *mio*-inosytol, itaconic acid, and tryptophane. The activity of induced extracellular proteolitic enzymes was higher among the very good colonisers and they were also tolerant to higher concentrations of coffeic acid.

Key words: *Pseudomonas*, wheat colonisation, physiological activity

Introduction

The ability of PGPR strains to colonise the rhizosphere is one of the main factors that determines the effectiveness of biocontrol (Lugtenberg & Dekkers, 1999). It is commonly known that certain physiological traits can affect the root colonisation by the *Pseudomonas* strains. They are : the ability to move towards the plant and along its root to settle down in an appropriate ecological niche, the attachment of bacterial cells to the root surface, the utilisation of the plant root exudates, the resistance to biological active components of root exudates and the ability to survive in the presence of competitive indigenous rhizosphere microflora (Gould, 1990). Despite of the importance of each of these processes, the detailed mechanisms involved in their efficacy are still poorly understood.

Material and methods

Colonisation of wheat seedlings

Forty *Pseudomonas* spp. strains were tested for their ability to colonise wheat seedlings. Suspensions of ~ 10^8 c.f.u. per ml of 48-hour-old bacteria were used for the bacterisation of the superficially disinfected wheat seeds. Seeds were incubated in pots with nonsterile sand at 28°C. To remove the bacteria seedlings were placed in 0.1 M solution of $MgSO_4$ and than sonificated. The number of c.f.u. on Gould's medium (Gould et al., 1985) was determined before as well as 24 and 48 hours after seeds planting. Strains were grouped with the application of the cluster analysis (Euclidean distance, method of full bonding) using Statistica for Windows version 5.1.

Ability of bacteria for utilisation of the root exudates

The utilisation of the organic compounds as a single carbon and energy source by *Pseudomonas* isolates was determined on mineral Stanier's medium (Stanier et al., 1966) containing 0.1% of one of the following organic compounds: glucose, arabinose, fructose,

sucrose, ribose, sorbitol, *mio*-inozytol, oxalacetic acid, fumaric acid, caffeic acid, ferulic acid, *trans*-cinnamic acid, *o*-cumaric acid, *p*-cumaric acid, malic acid, citric acid, succinic acid, bensoic acid, aconite acid, tartric acid, glutaric acid, glutaminic acid, aspartic acid, γ-aminobutyric acid, α-and β-alanine, glycine, serine, arginine, glutamine, valine, leucine, 1,2-propylenoglikol, histidine, tryptophan and trehalose. The bacterial growth on this medium was compared with the growth on control mineral Stanier's medium (without carbon source) and King's B medium after 3 days. Additionally, Biolog GN2 test was also used. Statistical analysis of these data was performed with the aid of the cluster analysis (percentage incompatibility, Ward's amalgamation algorithm) using Statistica for Windows version 5.1.

Ability of bacteria for growth in presence of phenolic acids

The ability of bacteria for growth in the presence of phenolic acids was tested on King's B medium (King et al. 1954) and on mineral Stanier's medium with glucose as a carbon source supplemented with *o*-cumaric acid, *p*-cumaric, caffeic acid, ferulic acid or *trans*-cinnamic acid in concentration from 0.01% to 0.1%.

Determination of the extracellular enzyme activity of bacteria

The activity of the proteolitic enzymes was analysed according to the Bradford's method (Bradford, 1976) in culture supernatants after 48-hrs of growth on liquid King's B or Stanier's medium with glucose. The activity of ß-glucosydase (Hoffmann & Dedeken, 1965), asparaginase (Johnson, 1941) as well as maltase and saccharase (Hofmann & Pallauf, 1965) were tested in supernatants from 7-days old cultures grown on liquid SRS (synthetic medium that mimic organic root secretions) (Gottlieb, 2001).

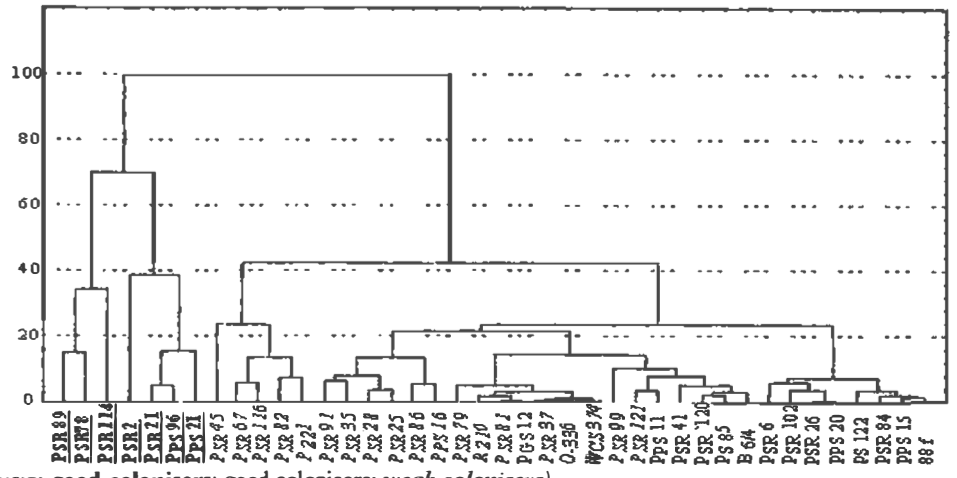
Determination of the antibiotic activity of bacteria

The ability to secrete antibacterial and antifungal antibiotics on King's B or SRS medium was determined by well method.

Results and discussion

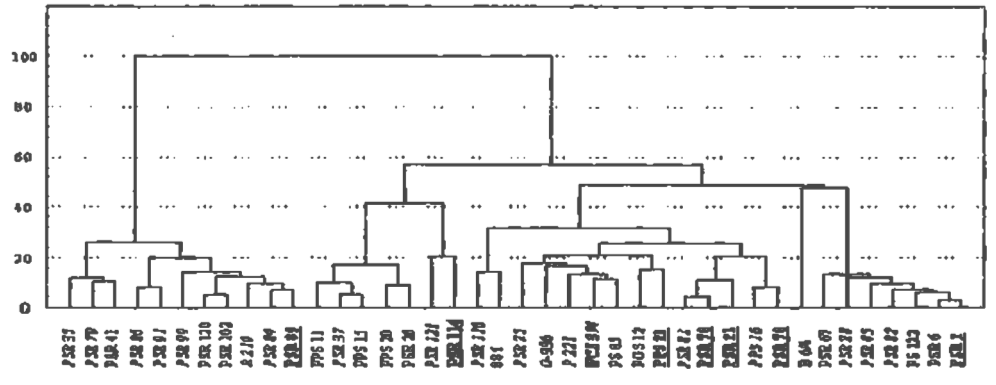
The *Pseudomonas* strains were classified into three groups in accordance to their ability for multiplication on wheat seedlings. Six strains showed the ability of significant increase of the number of viable cells from 3 x 10^5 c.f.u. per seed directly after inoculation up to 1 x 10^7 c.f.u. per seedling and were classified as very good colonisers. Strains which exhibited the ability to increase the number of viable cells from 1 x 10^5 c.f.u. up to 2 x 10^6 c.f.u. per one seedling were classified as good colonisers. Other strains of tested bacteria were classified as weak colonisers. Statistical analysis showed that very good colonisers created clusters dissimilar to all other bacteria. Remaining bacteria distinguished between themselves in a smaller extent (Figure 1).

The analysis of the capability for utilisation of organic compounds as single carbon and energy source revealed the lack of significant differences between these groups (Figure 2). However, the very good colonisers are characterised by metabolic superiority in utilising larger quantity of compounds. They were able to utilise the following compounds that were found in wheat root exudates: D-galactouronic acid, D-glucouronic acid, ferulic acid and *p*-coumaric acid to a larger extent than the remaining bacteria. They were also capable of utilising compounds not reported in wheat root exudates such as adonitol, *p*-hydroxyphenylacetic acid, *mio*-inosytol, itaconic acid, and tryptophan.



(very good colonisers; good colonisers; *weak colonisers)*

Figure 1. The cluster analysis of *Pseudomonas* spp. strains performed on the basis of the number of c.f.u. on the surface of 24 & 48 hrs old wheat seedling (Euclidean distance, method of full bonding).



(very good colonisers; good colonisers; *weak colonisers)*

Figure 2. The cluster analysis of *Pseudomonas* spp. strains performed on the basis of the ability for utilisation of organic compounds as a single carbon and energy source (percentage incompatibility, Ward's amalgamation algoritm)

The enzymatic activity of tested rhizobacteria showed that the biosynthesis level of induced extracellular proteolytic enzymes was higher among the very good colonisers. This kind of activity was found among 71% of these strains, but only among 36% of the good colonisers and among 39% of the weak colonisers. The activity of asparginase, β-glucosydase, maltase, saccharase and constitutive proteases was not significantly differentiated among tested bacteria.

Most of tested strains produced certain antibiotic compounds on liquid King's B medium. However, only two strains were ably to synthesise detectable amounts of antibiotic substances on SRS medium. We did not observe a correlation between antibiotic activity and seedling colonisation.

Analysis of physiological traits revealed that the very good colonisers were tolerant to higher concentrations of coffeic acid than the remaining groups. MIC of coffeic acid for these isolates came to 0.68 mg per ml. But for the good colonisers and weak colonisers MIC was 0.49 and 0.54 mg, respectively. The presented higher tolerance of the good colonisers to coffeic acid, an important plant metabolite of phytoalexins pathways, suggests that resistance for natural plant defence system could play a crucial role in the multiplication processes of bacteria in the rhizosphere.

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Discrimination of Czech *Armillaria* species based on PCR method and high performance liquid chromatography

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Abstract: The genus *Armillaria* belongs to basidiomycetes and has been known to induce root rot disease and to cause extensive economic losses to a forest crop. We analyzed about 40 isolates of *Armillaria* collected in Czech Republic by PCR and restriction analysis using gel electrophoresis and ion-exchange HPLC. Restrictase Hinf I was able to discriminate all *Armillaria* species. The sensitivity and resolution of HPLC method was better than that performed by gel electrophoresis. HPLC was able to detect some heterozygous. The results prove the similarity of the species *A. borealis, A. cepistipes, A. gallica, A. ostoyae* in difference of *A. mellea* and *A. tabescens*.

Key words: *Armillaria*, PCR, forest pathogen, basidiomycete, root rot disease

Introduction

The genus *Armillaria* belongs to basidiomycetes and has been known to induce root rot disease and to cause extensive economic losses to a forest crop. The main function of this fungus in the ecological system is the decomposition of wood waste, but it can very often turn to the necrotrophic parasitism and attack a wide range of woody species. Recently, the seven species of *Armillaria* have been identified in Europe: *A. borealis, A. cepistipes, A. ectypa, A. gallica, A. mellea, A. ostoyae* and *A. tabescens*. These species have different pathogenic behavior and thus the forest management necessitates confirming the presence of virulent *Armillaria* species. The current identification of *Armillaria* has included observation of cultural characteristics, pairing tests based on both sexual and somatic incompatibility, isozyme analysis and immunological techniques. Recently, molecular-biological technique has been used to identify *Armillaria* species. This technique provides very good reproducibility and the analysis is very rapid.

The aim of this study was to introduce the molecular-biological techniques of *Armillaria* species identification to the laboratory practice in Czech Republic and either confirm or revalue the previous classification of that species collected by Phytopathological laboratory of Masaryk University in Brno and Mendel University of Agriculture and Forestry Brno. The results and sequences of ITS region were compared with the published data.

Material and methods

Isolates of *Armillaria* strains were obtained from the collection of Department of Forest Protection, Faculty of Forestry, Mendel Agriculture and Forestry University of Brno and as a

generous gift of Collection of Microbiological Institute of Czech Academy of Sciences, Prague. Some isolates were a gift of dr. Guillaumin, INRA Clermond-Ferrand, France. We analyzed about 40 mycelia strains (number of isolates in parentheses): *A. borealis* (3), *A. cepistipes* (7), *A. gallica* (8), *A. mellea* (5), *A. tabescens* (6), *A. ostoyae* (10). The strains were previously identified by pairing tests.

DNA from mycelium was isolated using Kit Nucleospin plant (Macherey-Nagel). Amplification of ITS region was carried out by PCR using primers ITS1-ITS4 in a cycler Techne (Progene) and conditions described by White et al. (1990). The restriction analysis was performed with restrictases Alu I, Hinf I and Mbo I (Fermentas). The restriction fragments were analyzed in 3% agarose gel or using HPLC (HP 1100 Series) on the ion exchange column TSK-gel DEAE-NPR (Supelco) with a gradient of 0.3 - 0.8 M NaCl in 10 mM Tris/HCl (pH 9.0) during 10 min followed by 2 min washing period with 0.8 M NaCl (Katz & Dong, 1990). For sequencing, DNA was purified by precipitation in polyethyleneglycol and sequences using ITS1 and ITS4 primers in Laboratory of functional genomics, Masaryk University, Brno.

Results and discussion

The amplification of isolated DNA gave rise to the amplificates of 870 bp. The amplificates were submitted to restriction analysis. The resulting fragments were analysed either on agarose gel or using HPLC. The resolution and sensitivity of HPLC were better than those obtained by classical electrophoresis stained with ethidium bromide. We are able to detect 380 ng of DNA and resolution of peaks was better than 3% for the fragments of 500 bp. The raise of temperature from 22 to 26°C during HPLC increased the retention time of longer fragments by 2 min. The results of the restriction analysis of all *Armillaria* strains were compared with data published earlier by Chillali et al. (1998a, 1998b) and Schmidt (2001). Three types of results were obtained: 1) restriction fragment analysis corresponded to published sequence data, 2) the results corresponded to the other species so that the classification obtained by classical pairing test had to be revised, 3) the fragments profiles presented some anomalies, i.e. presence of additional bands thanks to a mutation in a restriction site.

Figure 1 shows a typical result of the HPLC analysis compared with gel electrophoresis of *A. cepistipes* 207 (restrictase Hinf I). The length of typical restriction fragments should be 40, 43, 103, 132, 227 and 315 bp. The figure shows a splitting of the peak of 132 bp into two fragments of 61 and 71 bp. The retention times are proportional to the base number similarly as molar absorption coefficients of the fragments. It is evident that HPLC detected only one half of the quantity of the fragments of 132, 61 and 71 bp so that the strain 207 must be a heterozygous. The restrictase HinfI enabled the separation of all species, whereas the restrictase AluI did not enable the discrimination of *A. borealis, A. cepistipes, A. gallica* and *A. ostoyae*. The restrictase MboI did not discriminate the species *A. gallica* and *A. cepistipes*.

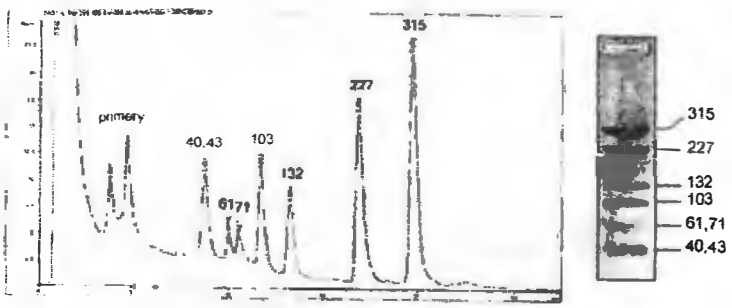

Figure 1. Restriction fragment analysis (Hinf I) of amplificate of A. *cepistipes* 207

As mentioned above, the restriction analysis of some species proved the presence of additional bands or disappearance of other restriction fragments caused by mutation or deletion in a restriction site. For this purpose, we proceeded to the sequencing of ITS region. Figure 2 shows polymorphism of ITS region of several isolates. The sequence homology into one species of all sequenced strains was 98-99%. However, the homology between *A. gallica* E4 (Finland) and *A.gallica* (Schmidt, 2001) and that between *A. mellea* (France) and *A. mellea* (Schmidt, 2001) was only 97%.

```
                    52 74 77 9  152 154 170 222 256 319 449 454 463 574 598  633
A.cepistipes :  34 ---t--a--g--t--t---c---c---a---X---g---c---g---t---g---c---ga-
A.cepist. 207:  34 --c--a--t--t--c---y---t---a---c---g---c---r---c---a---a---ga-
A.cepist. 182:  34 ---t--r--g--k--y---g---y---a---c---s---y---g---y---a---c---rr-

                    649 675 700 789
A.cepistipes :  --t---c---t---a---  868
A.cepist. 207:  --t---y---y---r---  838
A.cepist. 182:  --k---              670

                    43  222 240 464 467 474 485 512 636 677 725 745 759
A.gallica    :  26 --a---t---t---g---g---t---t---c---g---c---t---a---t---  871
A.gallica 173:  26 -------t---t---g---g---X---t---c---a---t---c---t---c---  841
A.gallica E4 :  26 --------c---a---t---c---X---g---g---g---t---c---t---c---  817

                    41 46 58 9  100 105 147 169 270 400 442
A.mellea     : 28 --a--g--a--t--X---g---a---g---X---c---a---  459
A.mellea  B2: 28 --t--c--g--a---g---t---g---a---c---X---t---  459

                    248 257 300 342 497 499 555 558 589 603 608 702 708 725 763
A.ostoyae    :  26 --c---t---g---t---g---g---t---t---t---t---a---c---g---g---g 871
A.ostoyae 151:  26 --y---y---g---k---g---t---t---t---t---t---a---s---r---g---r 829
A.ostoyae C2 :  26 --c---t---g---t---g---a---t---t---t---t---a---c---g---a--   745
A.ostoyae 209:  26 --c---t---s---t---r---g---k---w---w---k---m--               624

R = A/G, Y = C/T, M = A/C, K = G/T, S = G/C, W = A/T, X = deletion
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Figure 2. Polymorphism of ITS region of *Armillaria* species. All strains were collected inCzech territory whereas *A. gallica* E4 and *A. mellea* B2 in Finland and France, respectively. The sequence of strains marked by (*) was obtained from database (Schmidt, 2001)

The sequence homology between the strains are shown in Table 1. The results prove the similarity of the species *A. borealis, A. cepistipes, A. gallica, A. ostoyae* in difference of the strains *A. mellea* with the sequence homology of only 87-91% with all other species and *A.*

tabescens with 87 - 94%. Moreover, the sequence homology between *A. tabescence* and *A. mellea* was only 87%.

Table 1. Sequence homology of ITS region of *Armillaria* species. The number of isolates sequenced in parentheses.

	borealis	cepistipes (3)	gallica (3)	mellea (2)	ostoyae (4)	tabescens
borealis		98	97	91	98	89
cepistipes (3)	98		98	90	98	94
galica (3)	97	98		90	97.5	92
mellea (2)	91	90	90		91	87
ostoyae (4)	98	98	97.5	91		94
tabescens	89	94	92	87	94	

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In vitro relationships between microorganisms used as biocontrol agents

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Abstract: The relationships between the antagonistic fungi *Trichoderma viride*, isolates Td50 and Tv, and *Trichothecium roseum* (Tr) and other antagonistic bacteria (*Bacillus* sp.), fungi (*Trichothecium roseum, Gliocladium roseum, Coniothyrium minitans, Epicoccum purpurascens, Verticillium tenerum, Chaetomium* sp.) and entomopathogenic fungi (*Paecilomyces fumosoroseum, Beauveria bassiana*) have been studied *in vitro*. All the micromyceta in study have been inhibited *in vitro* by *T. viride*, with exception of *Epicoccum purpurascens*. Two *Bacillus* sp. strains (B48, B001) and one of *Pseudomonas* sp. (P12) significantly inhibited the growth of *T. viride*. These results lead to the conclusion that the different microorganisms with biocontrol abilities should be tested together before using them as a mixture, in order to be applied alone or in combination in integrated protection of plant diseases.

Key words: antagonistic microorganisms, *Bacillus* spp., *Pseudomonas* spp., *Trichoderma viride*, *Epicoccum purpurascens*, *Trichothecium roseum*, *Gliocladium roseum*, *Coniothyrium minitans*, *Verticillium tenerum*, entomopathogenic fungi, *Beauveria bassiana*, *Paecilomuces fumosoroseus*

Introduction

The paper presents *in vitro* relationships between the antagonistic bacteria (*Bacillus* spp., *Pseudomonas* spp.), antagonistic fungi (*Trichoderma viride*, *Epicoccum purpurascens*, *Trichothecium roseum*, *Gliocladium roseum*, *Coniothyrium minitans*, *Verticillium tenerum*, *Chaetomium* sp.) and entomopathogenic fungi (*Beauveria bassiana*, *Paecilomyces fumosoroseus*). The aim of the experiment was to establish the compatibility between these microorganisms in order to be used together in the sustainable protection of cropped plants against economically significant pathogens.

Material and methods

Biological material used consisted of 70 isolates of antagonistic bacteria and fungi belonging to the genera and species: *Bacillus* (19), *Pseudomonas* (24), *Trichoderma viride* (Td 50, Tv), *Epicoccum purpurascens* (Ep.), *Trichothecium roseum* (Tt 1, Tt 2, Tr), *Gliocladium roseum* (Gl 1, Gl 2, Gl 3), *Verticillium tenerum* (Vert. ten.), *Chaetomium* sp. (Chaet.), *Coniothyrium minitans* (7) and enthomopathogenic fungi *Beauveria bassiana* (Bb) and *Paecilomyces fumosoroseus* (Pf). The method of double culture *in vitro* has been used (Jouan et al., 1964; Bajan, 1978; Şesan, 1985; Constantinescu, 2000). Scoring the antagonistic fungal ability was done by calculating the ratio (coefficient) x between the inner (i) and outer (e) radius of the test-fungus (A) and the antagonists (B) after 5 days, with the following formula x = iA / iB x eB / eA. Scoring the antagonistic bacterial and entomopathogenic fungal abilities was appreciated by measuring the inhibition zone of test-microorganisms caused by the performed antagonist.

Results

Among 19 *B. subtilis* isolates tested against the test fungus *T. viride* (Td 50), B48 and B001 produced a maximum inhibition zone (4 mm) after 72 hours (hrs), followed by B38, B30, B36, B45a and B005 (3 mm). After 120 hrs, only 3 isolates (B001, B005, B30) preserved their antagonistic ability; B005 produced a well-delimited inhibition zone while B48 and B49 generated a limitation of *T. viride* mycelial growth. The smallest inhibition zone (2mm) has been produced by B57, B49 and BC1C isolates (Table 1).

Table 1. *In vitro* relationships between *Bacillus subtilis* and *Trichoderma viride* (Td 50)

No.	Isolate code	Inhibition zone (mm) after:		No.	Isolate code	Inhibition zone (mm) after:	
		72 hrs	120 hrs			72 hrs	120 hrs
1	B12	0	0	11	B48	4	limitation
2	B26	0	0	12	B49	2	limitation
3	B38	3	0	13	B001	4	3
4	B1.98	0	0	14	BC1C	2	0
5	B98	0	0	15	B45a	3	0
6	B57	2	0	16	B45c	1	0
7	Blob1	0	0	17	B005	3	3
8	B30	3	2	18	Bce2	0	0
9	Bsal	0	0	19	Control, Td50	70	70
10	B36	3	0				

Among 24 *Pseudomonas* isolates tested the strongest antagonistic activity was produced by isolate P12 (15 mm inhibition zone), followed by isolates P85 and P13 (12 mm). Other isolates (P26, P17, P20) proved a good antifungic activity expressed by an inhibition zone of 7-8 mm. The smallest inhibition zone (1-5 mm) has been caused by the isolates P9s, P17s and P18 (Table 2).

Table 2. *In vitro* relationships between *Pseudomonas* sp. and *Trichoderma viride* (Td 50)

No.	Isolate code	Inhibition zone (mm) after 72 hrs	No.	Isolate code	Inhibition zone (mm) after 72 hrs
1	P7s	0	14	P19s	0
2	P8s	10	15	P20	6
3	P9s	5	16	P20s	0
4	P12	15	17	P26	7
5	P13	10	18	P27s	0
6	P14	0	19	P1p	0
7	P15s	0	20	P2p	0
8	P16	0	21	P1v	0
9	P17	8	22	P2v	0
10	P17s	5	23	P5v	0
11	P18	1	24	P6v	0
12	P18s	0	25	Control, Td50	70
13	P19	0			

Using *T. viride* (Td 50) as a test fungus for screening other fungal isolates, the results proved the strongest antagonistic capacity of this most spread biocontrol agent of inhibiting the development of all performed potential antagonists (Table 3).

Table 3. *In vitro* relationships between *Trichoderma viride* (Td 50) and other antagonistic fungi, expressed by coefficient x

No.	Isolate code	X	Score[a)]	No.	Isolate code	x	Score[a)]
1	Tt 1	1.100	N	8	CR	1.200	N
2	Tt 2	1.100	N	9	C 15	1.300	N
3	Gl 1	1.100	N	10	C 18	1.860	N
4	Gl2	1.050	N	11	C 102	1.680	N
5	Gl 3	1.060	N	12	IVT 1	1.260	N
6	Vert. ten.	1.800	N	13	IVT 5	1.320	N
7	Chaet.	1.000	I	14	Ep.	0.990	SA
				15	Control (Td/Td)	1.000	-

a) x = 1 – absence of reciprocal influences between fungi (I = indifferent); x > 1 – antagonism absent (N); x<1 – the strongest antagonism the lower values are (A); SA = slight antagonist.

Table 4. *In vitro* relationships between entomopathogenic and antagonistic fungi, simultaneous inoculated, expressed by the fungal colony diameter (mm)

Fungus	Control	Bb/Tv	Bb/Tr	Pf/Tv	Pf/Tr	Pf/Bb
		(24 h)				
Beauveria bassiana	4	5	4	-	-	5
Paecilomyces fumosoroseus	5	-	-	1 .	5	4
Trichoderma viride	14	14	-	19	-	-
Trichothecium roseum	5	-	4	-	5	-
		(48 h)				
Beauveria bassiana	13	055	13	-	-	14
Paecilomyces fumosoroseus	2	-	-	1	19	21
Trichoderma viride	46	45	-	45	-	-
Trichothecium roseum	1	-	11	-	13	-
		(72 h)				
Beauveria bassiana	25	55	29	-	-	22
Paecilomyces fumosoroseus	33	-	-	1	41	31
Trichoderma viride	-	46	-	45	-	-
Trichothecium roseum	22	-	21	-	21	-
		(96 h)				
Beauveria bassiana	31	55	33	-	-	23
Paecilomyces fumosoroseus	40	-	-	1	55	56
Trichoderma viride	-	46	-	45	-	-
Trichothecium roseum	27	-	25	-	21	-

Legend: Bb = *Beauveria bassiana*; Pf = *Paecilomyces fumosoroseus*; Tv = *Trichoderma viride*; Tr = *Trichothecium roseum*

Investigations on the influence of antagonistic fungus *T. viride* (Tv) and two entomopthogenic fungi (Table 4) proved that *T. viride* developed a high inhibitory effect on *B. bassiana* and *P. fumosoroseus*, whose growth was impeded and covered by the antagonistic fungus. In comparison with *T. viride*, *T. roseum* showed a slightly inhibiting effect of the entomopathogens growth, even it grew faster than *B. bassiana* and *P. fumosoroseus*.

These data confirm our previous results (Şesan & Oprea, 1997, Andrei et al., 2002), for establishing the relationships between different microorganisms with practical importance in the biocontrol of plant diseases and pests. The results showed the following:

- Among 19 tested *Bacillus subtilis* strains against the test fungus *T. viride* (Td 50), B48 and B001 produced a maximum inhibition zone after 72 hours, and B57, B49, and BC1C a minimum one.
- From the 24 *Pseudomonas* isolates, the strongest antagonistic activity was produced by P12 strain, and the lowest by P9s, P17s, and P18.
- *T. viride* (Td 50), the test fungus used for screening other fungal isolates, proved the strongest antagonistic activity inhibiting the growth of all performed potential antagonists, with exception of *Epiccocum purpurascens*.
- *T. viride* (Tv) tested against *Beauveria bassiana* and *Paecilomyces fumosoroseum* showed a high antagonism in comparison with *T. roseum* (Tr).
- Based on the previous conclusion BCA's with strong antagonistic capacity against other BCA's should be used alone in biocontrol, while the synergic ones can be used together in a combined product in integrated protection of plant diseases.

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Soil-borne fungi and host plant influence on the efficacy of *Bacillus subtilis* biocontrol agents

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Abstract: Five *Bacillus subtilis* strains were tested against *Rhizoctonia solani*, *Pythium debaryanum*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum* and *Sclerotium bataticola* in two crops: tomato (Unirea var.) and cucumber (Comichon var.). The experiments were performed in greenhouse conditions in soil consisting of a mixture of sand, loam and manure. The results showed a biocontrol efficacy variation of the same strain for each fungi in function of the attacked crop. In the tomato crop, the highest efficacy against *Rhizoctonia solani* was reached in the treatment with Bs 98; against *Fusarium oxysporum* with Bs 30 treatment; against *Pythium debaryanum* and *Sclerotinia sclerotiorum* with Bs lob1 treatment and against *Sclerotium bataticola* with Bs sal and Bs 30 treatments (where the efficacy reached 100%). In the cucumber crop, consistant results were found for all the soil-borne fungi, the highest efficacy being reached in the variant with Bs 30 treatment. This behaviour of the *Bacillus subtilis* strains could be explained by different colonization of the plant rhizosphere and the impact on the resistance mechanism. Further experiments are necessary.

Key words: *Bacillus subtilis*, biological control, soil-borne fungi

Introduction

The most important mechanisms used by *Bacillus* spp. in the biocontrol of phytopathogenic microorganisms are: food competition, antibiotic production and induced resistance. The best function of these mechanisms and implicit the appearance and development inhibition of plant diseases is mainly influenced by environmentaly factors, including soil characteristics, plant species and rhizosphere microflora. Several studies (Bochow, 1998) revealed that the metabolites which are produced by *B. subtilis* induce resistance/ tolerance of vegetable seedlings to biotic and abiotic stress factors. Starting from our previous *in vitro* investigations on the biocontrol of 5 soil-borne fungi using *B. subtilis* strains, we continued with antibiotic extraction followed by *in vivo* screening in tomato and cucumber crops.

Materials and methods

Microorganisms and plants

Five *B. subtilis* strains (from R.I.P.P. collection) and five soil-borne fungi: *Rhizoctonia solani*, *Pythium debaryanum*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum* and *Sclerotium bataticola* were selected for the experiment. The bacterial inoculum was refreshed in tubes on PDA media (incubated at 27°C for 2-3 days) and used for seed inoculation as suspensions at 10^8 c.f.u./ml. Inoculum of fungi was obtained by cultivation on oat seeds (double sterilised at 1 atmosphere for 20 minutes) and incubated at 25°C for 3-4 days.

The seeds of tomato (Unirea var.) and cucumber (Cornichon var.), desinfected for 20 minutes with hypochloride 1% and rinsed three times with water, were treated before sowing by immersion in bacterial suspensions 10^8 c.f.u./ml for 20 minutes.

Screening of* Bacillus subtilis *strains

For each plant, the bifactorial experiment consisted in 8 variants with three repetitions. Three controls were carried out: chemical (standard)- Tiradin 70 WP (4g/kg); C2-untreated and infected; C1-untreated and uninfected. The soil used consisted of a mixture of sand, loam and manure. The soil was mixed uniformly with the fungal inoculum and distributed in trays (32/24 cm). One week after sowing, the number of the healthy seedlings was noticed and after 4 weeks the efficacy of the bacterial treatment was calculated. In order to establish the plant growth promotion effect induced by bacterial strains, the total length of the plants was measured. The statistic analyse was performed with ANOVA program.

Results and discussion

In the tomato crop the highest efficacy was registered for Bs lob1 treatment against *Pythium debaryanum* and *F. oxysporum*; Bs 98 treatment against *R. solani* and *S. sclerotiorum*; Bs sal and Bs 30 treatments against *S. bataticola* (Figure 1). In cucumber the highest efficacy against all the tested fungi was obtained by the Bs 30 treatment (Figure 2).

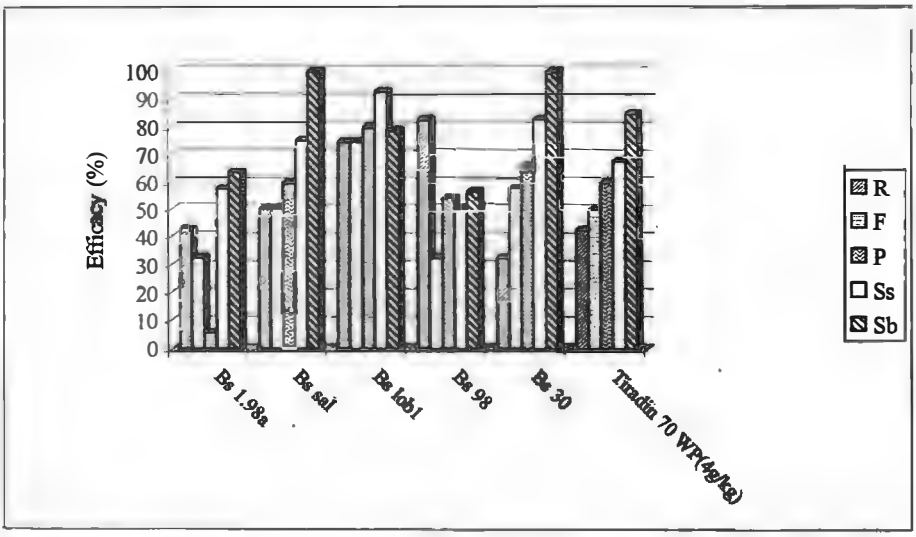

Figure 1. Efficacy of *Bacillus subtilis* strains in biocontrol of soil-borne fungi in tomato (Unirea cv.) under greenhouse conditions (R=*Rhizoctonia solani*, P=*Pythium debaryanum*, F=*Fusarium oxysporum*, S=*Sclerotinia sclerotiorum*, Sb=*Sclerotium bataticola)*

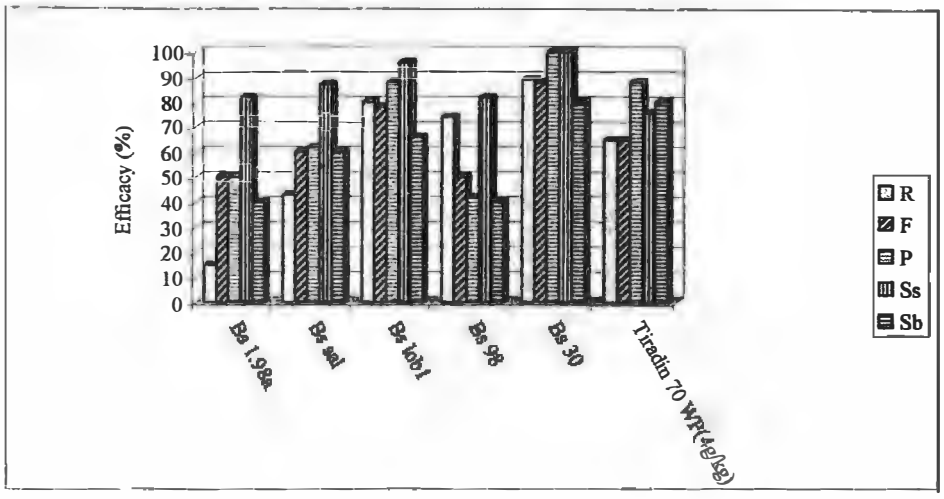

Figure 2. Efficacy of *Bacillus subtilis* strains in biocontrol of soil-borne fungi in cucumber (Cornichon cv.) under greenhouse conditions (R=*Rhizoctonia solani*, P=*Pythium debaryanum*, F=*Fusarium oxysporum*, S=*Sclerotinia sclerotiorum*, Sb=*Sclerotium bataticola)*

Assessment of the influence of the seed bacterisation on plant growth promotion was analysed statistically with ANOVA program. The results are presented in Figure 3 and 4. It was noticed that for tomato, the highest total length was reached by Bs lob1 and Bs 30 treatment. In thecucumber crop, compared to the untreated – infected control, length of the plants was significantly increased by Bs 30.

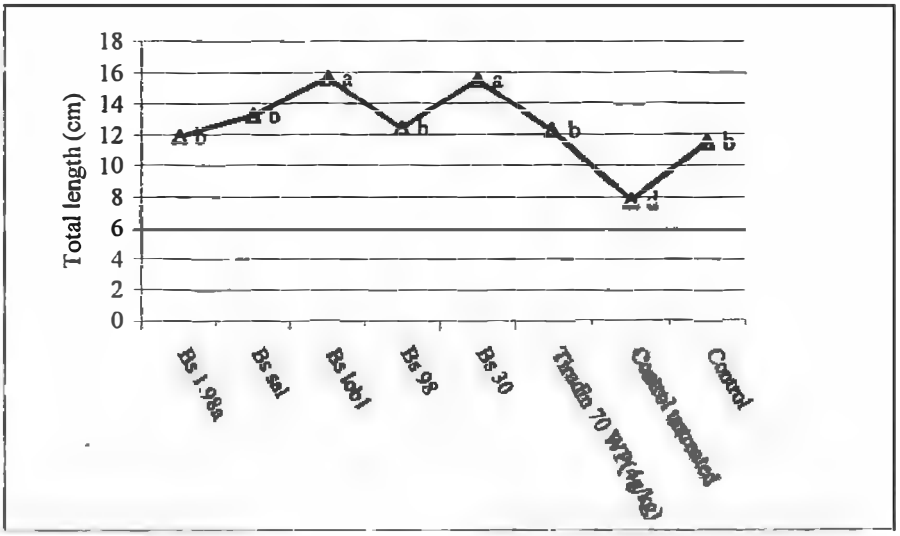

Figure 3. Effect of *Bacillus subtilis* strains on tomato plant growth

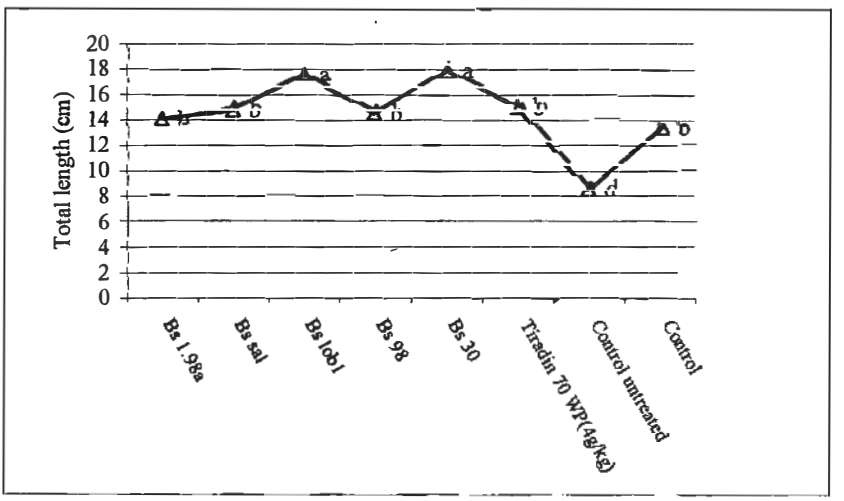

Figure 4. Effect of *Bacillus subtilis* strains on cucumber plant growth

These results lead to the conclusion that *B. subtilis* strains which promoted the plant growth were also good biocontrol agents against the soil-borne fungi studied. The mechanism of biocontrol showed positive correlation with this property.

There was no positive correlation between *in vitro* and *in vivo* results, regarding the antagonistic capacity of the bacteria.

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Environmental factors and screening of better biocontrol agents

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Importance of environmental conditions during antagonists selection for biocontrol of toxigenic *Fusarium* spp. in wheat

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Abstract: *Fusarium culmorum* and *F. graminearum* can cause head blight in cereals, resulting in lower yields and kernels containing mycotoxins. Ninety-seven candidate antagonists have been tested for their reduction in sporulation of *Fusarium culmorum* and *F. graminearum* on wheat straw, which can act as primary inoculum source of *Fusarium*. When tested in the initial screening, at 15 °C and continuously high humidity, 21% of the antagonists were able to reduce sporulation of *F. culmorum* and/or *F. graminearum* for 90% or more as compared to the water control. However, when tested against *F. culmorum* under fluctuating temperature and humidity conditions several of these isolates did not reduce sporulation. In contrast, a few other antagonists with moderate efficacy in the initial screening reduced sporulation significantly, up to 99.8%. These results stress the importance of implementing realistic micro-climatic conditions early in the screening process.

Key words: *Fusarium culmorum, F. graminearum*, antagonist, wheat, screening, bioassay, micro-climate

Introduction

Fusarium culmorum and *F. graminearum* can infect wheat and other cereals during flowering causing Fusarium head blight (ear blight, scab). *Fusarium* damaged kernels are shrivelled, resulting in low yields, and they can contain significant amounts of mycotoxins, e.g. deoxynivalenol (DON) or nivalenol, produced by *Fusarium* spp.. Occurrence of head blight depends on weather conditions during flowering and the amount of primary inoculum present in the crop. This inoculum is mainly produced on crop residues such as stubble. A screening programme has been started in the framework of EU-project ControlMycoToxFood (QLK1-1999-00996) to select antagonists reducing the risk of head blight by biologically eradicating inoculum sources of *Fusarium* spp.

Material and methods

Initial screening of antagonists on straw

Eighty-six fungi, nine yeasts and two bacteria have been tested for their capacity to reduce sporulation of *F. culmorum* and *F. graminearum* on straw. The candidate antagonists were grown for 14 days on PDA, 28 days for slow-growing fungi, yeasts on basal yeast agar for 5 days and *Fusarium* spp. on SNA (Nirenberg, 1976) for 14 days with 12 h blacklight per day.

Wheat straw was cut into segments, gamma-irradiated and soaked in sterile tap water before use. Three soaked segments were transferred to a moist chamber consisting of a Petri dish with moist filter paper. Subsequently, segments were inoculated with a conidial suspension of *Fusarium* spp. (10^4 conidia/ml), using sterile atomisers and incubated for 6 h at 15 °C under moist conditions in the dark. Thereafter, the following treatments were carried

out: water-control (sterile tap water containing 0.01% Tween 80); suspensions containing spores of different candidate antagonists (fungi and yeast: 10^6 spores or cells/ml, bacteria 10^7 cells/ml, both in 0.01% Tween 80). For both *F. culmorum* and *F. graminearum*, three replicate Petri dishes were used for each treatment with antagonists; for the water control treatment, six replicates were used. Petri dishes sealed with parafilm were further incubated at 15 °C with 12 h blacklight per day. Eleven different bioassays were carried out to test a total of 97 candidate antagonists.

The amounts of conidia of *F. culmorum* and *F. graminearum* produced on the straw segments were determined after 21 days. For direct counting, straw segments of each Petri dish are put into an Erlenmeyer containing 10 ml of a washing liquid (20% ethanol in water containing 0.01% Tween 80). Erlenmeyers were shaken for 10 min and conidial concentration of the suspensions were determined microscopically for *F. culmorum* and *F. graminearum* using a haemocytometer. The obtained numbers of conidia of *F. culmorum* or *F. graminearum* per replicate Petri dishes were log-transformed and analysed by ANOVA for each bioassay. LSD-tests ($\alpha = 0.05$) were carried out for separation of means.

Experiments in mist chambers

Climate cabinets with a computer-controlled misting facility were used to simulate micro-climatic field conditions. Segments of straw were fixed on vertically positioned sticks so that they had no contact with each other or the cabinet floor. Experiments lasted four weeks with daily cycles of 4 h at 8 °C with misting followed by 20 h at 18 °C without misting. Wetness periods in straw segments were monitored using leaf wetness sensors specially designed for monitoring leaf wetness within necrotic tissues. Under the experimental conditions, wetness periods in straw started immediately with the misting period at 8 °C and continued for approximately 8 h (Figure 1).

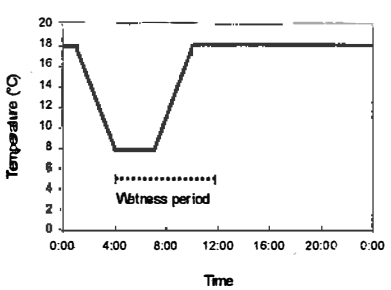

Figure 1: Fluctuating temperature and wetness period in mist chambers

Straw segments were inoculated with *F. culmorum* and (selected) antagonists as described above, before they were fixed in the mist chamber. Per replicate of each treatment, four segments were used. After four weeks, straw segments were placed in separate moist chambers for each replicate of each treatment and were incubated for three weeks at 15 °C with 12 h blacklight per day. Conidia of *F. culmorum* were subsequently washed off as described above. The obtained numbers of conidia of *F. culmorum* per replicate each consisting of four straw segments were log-transformed and analysed by ANOVA. LSD-tests ($\alpha = 0.05$) were carried out for separation of means.

Results

Initial screening

The 97 candidate antagonists showed a substantial difference in their ability to colonise wheat straw and compete with Fusarium spp.: reduction in Fusarium sporulation varied from 0% to 100% (Table 1). Twenty-one percent of the fungal isolates were able to reduce sporulation of *F. culmorum* and/or *F. graminearum* for 90% or more, as compared to the water control. The isolates did not necessarily suppress both Fusarium spp. to the same extend (Table 1, Figure 2).

Experiments in mist chambers

So far eleven antagonists, varying from moderate to high efficacy against both *Fusarium* spp. in an initial screening, have been tested under fluctuating conditions. Several isolates with high efficacy (> 90%) in the initial screening showed only moderate antagonism under fluctuating temperature and water availability; they reduced *F. culmorum* sporulation for 69 to 86 % (Figures 2 and 3). In contrast, some antagonists, which only showed moderate antagonism in the initial screening, suppressed *F. culmorum* sporulation almost completely under fluctuating conditions for up to 99.8 % (Figures 2 and 3).

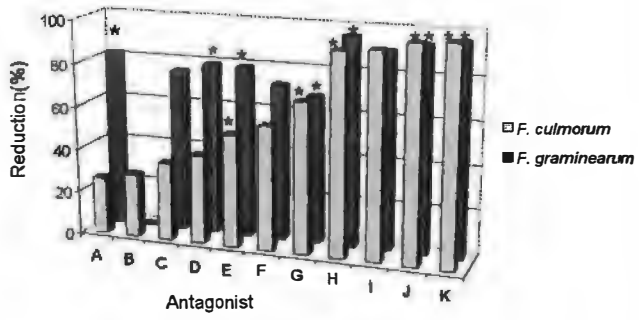

Figure 2. Percentage reduction in sporulation of *F. culmorum* and *F. graminearum* by selected antagonists under continuously most conditions at 15 °C; *: significantly different from water control in the various bioassays.

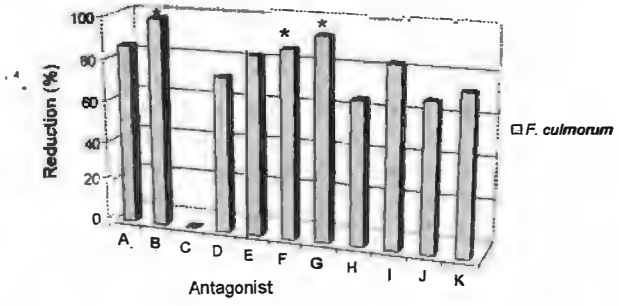

Figure 3. Percentage reduction in sporulation of *F. culmorum* by selected antagonists under fluctuating conditions: temperature 8 – 18 °C, wetness period during low temperature: 8 h wet - 16 h dry (Figure 1). *: significantly different from water control in various bioassays.

Table 1. The efficacy of antagonists to reduce sporulation of *F. culmorum* and *F. graminearum* on wheat straw under continuously moist conditions at 15 °C

	Number of antagonists in efficacy category	
Efficacy (%)	*F. culmorum*	*F. graminearum*
<5	9	20
5 - <50	49	17
50 - <90	19	40
90 - 100	20	20

Discussion

The *Fusarium* spp. are capable to survive saprophytically on crop residues. These residues are considered to be the most important reservoir for infection of wheat ears (Parry et al., 1995; Dill-Macky & Jones, 2000). Our results indicate that suppression of *Fusarium* sporulation on crop residues by antagonists is possible. The strategy of suppressing sporulation to minimise infection can also be used against necrotrophic pathogens in general (Köhl & Fokkema, 1998).

Our results also show that the outcome of biocontrol experiments strongly depends on environmental conditions. Knowledge of environmental conditions of the niche in which an antagonist will be introduced is essential for the choice of conditions for testing its ecological competence. Possible bottlenecks for antagonist performance such as low temperatures or interruptions of wetness periods should be considered early in a screening programme. Relying on data on antagonism assessed only under optimum conditions, or assessed on artificial media instead of natural substrate, may result in selecting antagonists, which are not efficient in the real world of crops.

Acknowledgement

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Pre-harvest biocontrol of Fusarium pathogens of maize

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Abstract: A collection of 60 potential antagonists (19 isolates from Italy, 10 from the Netherlands, 31 from U.K.) was screened against *Fusarium verticillioides*, *F. proliferatum*, *F. graminearum* and *F. culmorum*, using the maize stalks colonisation assay by spraying. Among the potential antagonists tested, 4 isolates showed a good antagonism towards all target *Fusarium* pathogens.

A field trial with maize stalk strips was performed to test the most promising antagonists towards *F. verticillioides*, *F. proliferatum* and *F. graminearum*, by spraying. Every two months, the strips are collected and development and survival for each combination *Fusarium*/antagonist is checked. Preliminary results showed a good reduction of development of *F. graminearum* by the antagonists tested. Two out of the four antagonists used in this experiment reduced the development of *F. verticillioides*.

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Biocontrol of cacao fungal diseases – example of disease management in a tropical tree crop

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Abstract: Growth characteristics of the fungus *Trichoderma stromaticum*, a mycoparasite on mycelium and fruiting bodies of *Crinipellis perniciosa*, the causal agent of the witches' broom disease of cacao (*Theobroma cacao* L.) was studied under controlled temperature (20, 25, 30 °C) and relative humidity (75% and 100% RH) to reflect conditions in Bahia, Brazil. Growth and sporulation of the mycoparasite was evident only under high relative humidity (100% RH) and temperatures of 20 and 25 °C, but not at high temperature (30 °C) or low RH (75%). Growth and sporulation of *T. stromaticum* completely eliminated *C. perniciosa* within the brooms, within 7 days of incubation. Results suggest that applying *T. stromaticum* under higher moisture conditions and when air temperatures are lower than 30 °C may enhance the establishment of this mycoparasite in cacao plantations. Currently we are developing an IPM schedule in which *T. stromaticum* will be applied during the cooler and wetter periods (June to August) of the year in Brazil. Research also includes formulating the mycoparasite to improve its colonization of the brooms. Field trials in Peru have shown that under lower disease pressure, spraying cacao trees with *T. stromaticum* results in reduction of disease symptoms on the pods, flowers and leaves.

Key words: *Theobroma cacao*, cacao diseases, biological control, *Trichoderma stromaticum*

Introduction

Infection of cacao by *Crinipellis perniciosa* has reduced cocoa production in Brazil by 75% in the last ten years. The pathogen infection results in pod loss and also leads to swelling and formation of numerous succulent vegetative branches known as brooms within flower cushions, vegetative apical and axillary buds. Basidiocarps are formed on these brooms and on pods, and these are the source of infective basidiospores. Managing witches' broom disease of cacao through biocontrol has been investigated for the past several years. A new species of Trichoderma, *T. stromaticum*, a mycoparasite on the witches broom (Bastos, 1988; Samuels et al., 2000) has been identified. *T. stromaticum* is a parasite on the mycelium and basidiocarps of *Crinipellis perniciosa*, causal agent of witches' broom disease in cacao. In Brazil, commercial formulations of *T. stromaticum* (TRICOVAB) are currently used in managing witches' broom. However, inconsistent performance of *T. stromaticum* indicates the need for further understanding of the relationship of the cacao plantation environment to the survival and establishment of this mycoparasitic fungus.

To optimize the use of *T. stromaticum* as a biocontrol agent, we are currently studying the effect of environmental factors on the survival, sporulation, and dissemination of *T. stromaticum* in cacao. The objective of this study was to assess the effect of temperature and relative humidity on the sporulation of *T. stromaticum* under controlled-environment conditions and also test the effectiveness of the biocontrol agent in the field.

Materials and methods

Sporulation of *T. stromaticum* on dry brooms of cacao was evaluated in relation to three constant temperatures (20, 25, and 30 °C) and two relative humidity levels (75, and 100%). Dry broom segments (4 cm long at 10 to 12% moisture content) were immersed in sterile distilled water to raise their moisture content to approximately 40%. They were then sprayed with a conidial suspension (10^6 conidia/ml) and placed in uncovered petri plates (4 segments per plate). The plates were inserted into humidity chambers containing water or saturated sodium chloride (NaCl) on the bottom. Water and saturated NaCl solution were used in the chambers to generate high (100%) and low (75%) relative humidity, respectively. The chambers were then maintained in incubators set at desired temperature levels. Sporulation on broom segments was evaluated after 2 weeks incubation.

Field sites have been established in Bahia, Brazil and in Apurimac valley in Peru to test the effectiveness of the biocontrol formulations in reducing the witches broom disease. The biocontrol agent was grown on solid substrates such as rice grains to obtain a conidial preparation of *T. stromaticum*, and in low-carbon molasses yeast extract medium mixed into BIODAC (an inert granular material made from newspaper waste) to obtain both conidial and chlamydospore preparation. Chlamydospore and conidial preparations (~ 10^7/ml) mixed with water were used to spray cacao trees. Motorized or back-pack sprayers were used to deliver approximately 250 ml to 500 ml of the formulations per tree. In Peru four applications of biocontrol agents were done every 30 days. In Brazil the application were done every 15 or 30 days for 3 months. Water sprays were used as controls.

Results and discussion

There was no production of conidia on broom segments at 75% relative humidity at any temperature. On brooms maintained at 100% relative humidity, *T. stromaticum* produced abundant conidia at 20 and 25 °C, but none at 30 °C. No mycelium of *C. perniciosa* was observed growing from brooms at 75% relative humidity. From brooms maintained at 100% relative humidity, mycelium of *C. perniciosa* grew at all temperatures, but not from brooms sprayed with conidia of the mycoparasite. *T. stromaticum* was readily recovered from broom segments maintained at 20 and 25 °C regardless of the relative humidity, whereas the fungus could not be recovered from segments subjected to 30 °C. When brooms maintained at 75 and 100% relative humidity and at 20 and 25 °C were remoistened and placed on moistened sand, sporulation was evident within four days at an incubation temperature of 25 °C. Results from field trials in Peru showed that *T. stromaticum* from Brazil was effective in suppressing witches' broom under lower disease pressure. Application of biocontrol agents reduced significantly disease on pods, stems and flowers. In addition to *T. stromaticum*, *T. viriens* (SG: Soilgard) and *Cladobotryum amazonense* (CA) were also used. Single strains were as effective as mixtures.

The most consistent effect was with the trees sprayed with *T. stromaticum*. In Brazil, rice formulations of *T. stromaticum* sprayed (10^8 CFU/ml) on the canopy reduced basidiocarp formation by 89%. A large percentage (85%) of these brooms were colonized by the biocontrol agent, which was tested by re-isolating the agent on PDA agar medium. On an average, biocontrol sprayings reduced basidiocarp formation from 131 basidiocarps/30 brooms to 30 basidiocarps when sprayed every 30 days, and 14 basidiocarps when sprayed every 15 days. Total basidiocarp production, in the control brooms were 395 as compared to 43 and 90, with 15 and 30-day sprays respectively. These treatments did not reduce witches broom infection of pods and flower cushions.

The main conclusion of this study is that *T. stromaticum* is effective at temperatures lower than 30 °C, with high capacity to sporulate under high moisture conditions. In Bahia, the major cocoa production area of Brazil, average maximum and minimum temperatures are well below 30 °C with relative humidity above 75% during the winter months (June to September). As part of an IPM schedule, applying this mycoparasite during the cooler months should increase the likelihood of establishment of *T. stromaticum* in cacao plantations. However, as the level of the disease is extremely high, better biocontrol agents are needed. In Peru, the control measures may have been more successful mainly due to lower witches' broom disease pressure than in Bahia and also be due to the different agro climatic conditions and lower rainfall.

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Influence of micro-organism isolation site (leaf and soil) on antagonistic activity against leaf (*Botrytis cinerea*) and root (*Armillaria mellea*) pathogens

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Abstract: The use of a single antagonist is not generally sufficient to give a total control of diseases in the field. An alternative for improving efficacy of antagonists could be the integration of several microorganisms with different action mechanisms on the pathogen. Isolation and testing for antagonistic activity is always a critical and time-consuming step in identification of useful microorganisms for biocontrol and finding effective antagonists is not an easy task. The aim of the research was to understand if there is a relation between isolation site (leaf and soil) and the amount of achievable antagonists against airborne or soilborne pathogens. Different isolation methods were also compared. Micro-organisms were isolated respectively from grape leaf surface or leaf material and rhizosphere or soil of some abandoned vineyards using different isolation techniques. Leaf microorganisms were isolated by washing, from crushed leaves, plant necrotic tissues and *Plasmopara viticola* sporulation areas. Soil micro-organisms were isolated in the rhizosphere, by the use of mycelium baits of a root pathogen (*Armillaria mellea*), directly from *A. mellea* mycelium, rhizomorphs and basidioma and from decayed roots. The isolates (134 from leaf and 123 from soil) were assayed in dual culture for its antagonistic activity towards two grape pathogens, *Botrytis cinerea* and *A. mellea*, used respectively as model for leaf and root pathogens. Antagonistic efficacy of microorganisms was evaluated as reduction of pathogen growth and action mechanisms of the effective ones were divided into three groups (antibiotic or toxin producing organisms, mycoparasites and resources competitors) depending on pathogen colony macroscopic alterations and microscope analysis. The results obtained with the different isolation methods are discussed.

Keywords: antagonist, isolation methods, *Botrytis cinerea*, *Armillaria mellea*

Introduction

The use of a single antagonist is not generally sufficient to give a total control of diseases in the field. An alternative for improving efficacy of antagonists could be the integration of several micro-organisms with different action mechanisms on the pathogen. Consequently several micro-organisms with different growing requirements and characteristics are needed. Isolation and testing for antagonistic activity is always a critical and time consuming step in identification of useful micro-organisms for biocontrol and finding effective antagonists is not an easy task (Campbell, 1989; Dhingra & Sinclair, 1985). Finding the best isolation methods and the appropriate materials could help in collecting effective ones.

The aim of the research was to understand if there is a relation between isolation site (leaf and soil) and the amount of achievable antagonists against airborne or soilborne pathogens. For this purpose two important in vitro culturable grape pathogens, *Botrytis cinerea* (grey mould) and *Armillaria mellea* (root rot), were used. Both of them are rotting pathogens that can survive also

as saprophytes. The former is a typical pathogen of green tissues and berries, the latter attacks only roots or woody tissues.

Materials and methods

Micro-organisms were isolated respectively from grape leaf surface or leaf material and rhizosphere or soil of 17 different vineyards located in the north-east of Italy. The vineyards were abandoned and untreated for at least three years. Micro-organisms were isolated on Potato Dextrose Agar (PDA) and Nutrient Sucrose Agar (NSA) and grown 20°C. Several materials were used in isolation trials: leaf washing water (WAS), necrotic leaf portions grounded in sterile water with mortar and pestle (GRD), necrotic leaf pieces (LFP), atypical and weak sporulation of in vitro unculturable grape pathogen (*Plasmopara viticola*) (SPR), micro-organisms collected from air (AIR), fresh pieces of *A. mellea* fruiting body (CRP), two weeks old *A. mellea* colonies left in the vineyard soil (20 cm deep) for 5 days (BAT), *A. mellea* mycelium coming from diseased roots (MIC), root surface material scraped with a lancet (RTS), healthy root pieces (RTP), old and partially degraded *A. mellea* rhizomorphs (RIZ) partially decayed woody material collected in soil (WDD). For each kind of material used, all morphologically different colonies of fungi and bacteria were collected.

A sample of 257 isolates (134 from leaf and 123 from soil material) was assayed in dual culture for its antagonistic activity towards two grape pathogens, *B. cinerea* and *A. mellea*, used respectively as models for pathogens of the aerial part of plant and roots. The putative antagonists and the pathogens were inoculated on suitable medium in Petri dishes (PDA or SNA) at a distance of 5 cm from each other. The radius, perpendicular to antagonist, (AC) of *B. cinerea* or *A. mellea* colony was measured when the opposite side of the colony was at least 2.5 cm long (AD). Antagonistic efficacy of micro-organisms was evaluated as reduction of pathogen growth (AD-AC).

Micro-organisms were divided into four classes:

very good with (AD-AC)*100/AD> 75%,
good with 50% <(AD-AC)*100/AD< 75%,
sufficient with 25% <(AD-AC)*100/AD< 50%,
insufficient with (AD-AC)*100/AD< 25%.

Depending on pathogen colony macroscopic alterations and microscope analysis, the putative action mechanisms of the effective ones were divided into four groups: antibiotic or toxin producing organisms (A), mycoparasites (M), resources competitors (S) and with no determinable mechanism of action (n.d.).

Results and discussion

Micro-organisms coming from soil material are more effective against *B. cinerea* than *A. mellea* under in vitro conditions. Vice versa those coming from leaf material are more effective against *A. mellea* than *B. cinerea* (Figure 1). 30.5 % of micro-organisms shows effects against both pathogens, 36.1 % only against *A. mellea* and 2.8 % only against *B. cinerea*. 30.5 % of them shows no effects on *A. mellea* or *B. cinerea* growth. In the studied vineyards differences in number and species of isolated micro-organisms are found (results not shown). Mycoparasites

(66.7 %) prevail among micro-organisms with very good effect against both *B. cinerea* and *A. mellea*.

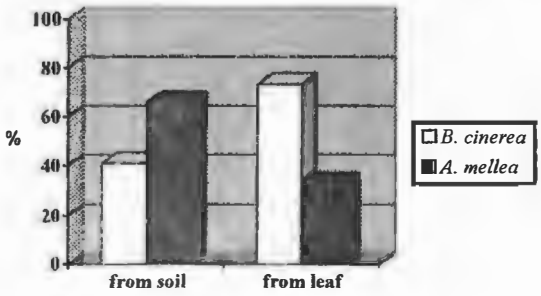

Figure 1. Percentage of micro-organisms isolated from soil and leaf material that shows antagonistic activity respectively against *B. cinerea* or *A. mellea*

The lowest percentage of antagonists is found in leaf washing water. None of the organisms isolated from *A. mellea* fruit body is active against *A. mellea* and only a small part of them shows some activity against *B. cinerea*. In *P. viticola* atypical sporulations are mostly found organisms with good activity as resource or space competitors, but they show low level of antagonistic activity. The proportion of antagonists among isolated micro-organisms is higher if rhizosphere or soil material is used (Figure 2).

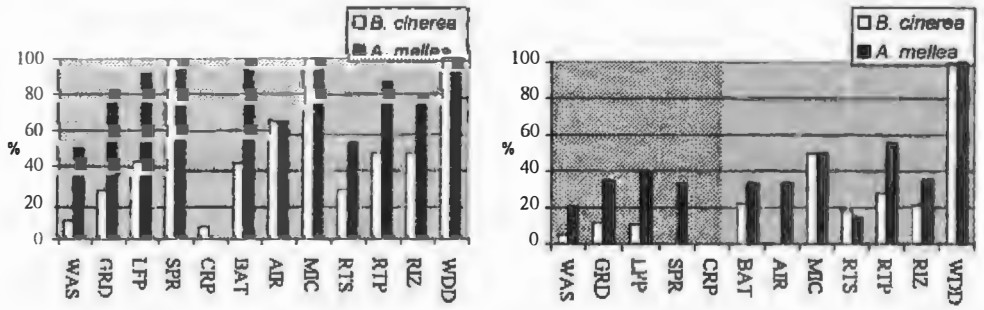

Figure 2. Percentage of micro-organisms with antagonistic activity against *B. cinerea* and *A. mellea* isolated from different soil and leaf material. All active antagonists (left), very good ones (right)

Mycoparasites are more frequently isolated from rhizosphere or decaying material. Antibiotic producers or space competitors prevail on leaf surface or tissues (Figure 3).

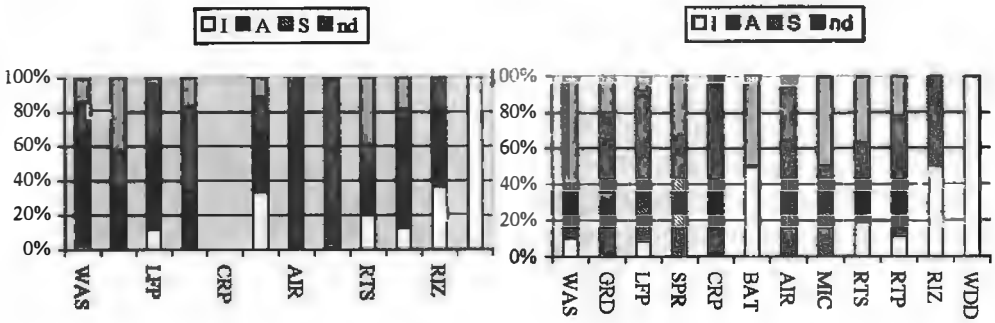

Figure 3. Proportion of organisms with different action mechanism against *A. mellea* (left) and *B. cinerea* (right) found in materials used for isolation.

The material used for isolating potential antagonists has a remarkable influence on the characteristics of obtainable isolates. Isolation methods must carefully planned and the material must be chosen according to the request antagonist qualities and the target pathogen.

In the present research micro-organisms coming from leaves show a better antagonism against *A. mellea*, on the other hand those coming from rhizosphere or soil material seem to be more effective against *B. cinerea*. *A. mellea* was present as grape root pathogen in many of the vineyards. Different results would probably be obtained with material coming from *A. mellea* suppressive soils and the antagonistic activity could be quite different in field conditions.

Among the tested organisms the most effective in vitro antagonists against both *B. cinerea* and *A. mellea* are the mycoparasites which are mostly isolated from soil and rhizosphere or partially degraded pathogen mycelium. A high antagonistic activity in vitro often results in low efficacy in field. More studies are needed to better characterise the isolates in natural conditions and to find the best application schedule in the vineyard against each grape pathogen.

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Effects of bioprotectants on seed pathogens, seed emergence and grain yield of wheat

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Abstract: Seed-borne pathogens are extremely important on wheat crop in Brazil. Despite its shortcomings as control measure, seed treatment with fungicides remains the most important and recommended tool these pathogens. Bioprotectants may play na important role in seed pathogens management on wheat in Brazil. Experiments were carried out under laboratory and field conditions in Passo Fundo, RS and in Pato Branco, PR, aiming to evaluate the effects of seed bioprotectants on seed pathogens, seed emergence, and grain yield of wheat. In the laboratory, most bioprotectants tested significantly reduced pathogen recovery from infected wheat seeds. In the field, all bioprotectors tested significantly improved seed emergence and wheat grain yield over nontreated control. The best bioagents were *Paenibacillus macerans* (007) P, *Pae. macerans*(007) WP, *Pantoea agglomerans* (1494) P, *Pan. Agglomerans* (1494) WP, and *Pseudomonas fluorescens* (4291) WP. Yield increase was up to 309 kg per hectare in the best treatment. Dressing wheat seeds with bioprotectants appears to be atractive technology for pathogen protection in Brazil.

Introduction

Seed-borne pathogens are extremely harmful organisms on wheat crop in Brazil (Luz et al., 1976; Luz, 1987). Despite its shortcomings as control measure, seed treatment with fungicides remains the most important and recommended tool for such pathogens. Bioprotectants may play an important role in seed pathogens management on wheat in Brazil (Luz, 1996).

Material and methods

Biological treatments were applied by shaking 500 g of seed with the appropriate rate of each treatment. Two experiments were performed in Passo Fundo, State of Rio Grande do Sul, Brazil. Treatments are shown in table 1. In the laboratory experiment, the presence or absence of pathogens was determined "in vitro" on PDA, 5 days after plating 100 seeds per treatment. In the field experiment, plots were 5 m in length by 4 m wide. Each row was 1 m apart and the seeds were sown 20 cm apart. The experimental design used was a randomized complete block with 4 replicates in the field and a complete randomized design with 4 replicates in the laboratory. The number of seedlings was counted in each plot. Plots were harvested and the grain yield recorded. Data were subjected to analysis of variance and means were separated using Fisher's least significant difference (LSD) test (P=0.05).

Results and discussion

In the laboratory, most bioprotectants considerably reduced pathogen recovery from infected wheat seeds (Table 1). All bioprotectants tested significantly improved seed emergence and wheat grain yield over the non-teeated control. The best bioagents were *Paenibacillus macerans* (007)P, *Pae. macerans* (007)WP, *Pantoea agglomerans* (1494)P, *Pan. agglomerans* (1494) WP, and *Pseudomonas fluorescens* (4291)WP (Table 2) . Yield increase was as high as 309 kg per hectare in the best treatment (Table 3). One of the mechanism by which these bioprotectants act is by antibiosis (Luz, 2001). Other studies using *Paenibacillus macerans* (144) and an isolate of *P. putida* biotype B for wheat seed protection against *D. tritici-repentis* (Luz et al., 1998) provided similar evidence. Beneficial effects of bioprotectants on plants have been previously reviewed (Bakker et al., 1991; Kloepper, 1991, 1993; Luz, 1993, 1996). Other mechanisms such as hidrocyanic acid, siderophores and induction of resistance may also play a role in the action of PGPR. *B. megaterium* performed better in Pato Branco, state of Paraná, than in Passo Fundo, state of Rio Grande do Sul. Dressing wheat seeds with bioprotectants appears to be an attactive technique for pathogen protection in Brazil.

Table 1. Effect of seed-applied bioagents on pathogen recovery from infected wheat seed

Treatments	% fungal recovery *		
	Dt-r	*F.g*	*S.n*
Untreated Check	8	6	11
Paenibacillus macerans(007)P	2	0	0
Paenibacillus macerans(007)WP	2	2	1
Pantoea agglomerans(1494)P	0	0	0
Pantoea agglomerans(1494)WP	0	0	0
Pseudomonas fluorescens(4291)WP	0	3	3
Pseudomonas fluorescens(4291)P	0	3	5
Bacillus megaterium(9790)P	6	2	3
Bacillus megaterium(9790)WP	0	2	4

Dt-r = Drechslera tritici-repentis, F.g = Fusarium graminearum, S.n. = Stagonospora nodorum.

Table 2. Effect of seed-applied bioagents on wheat seedling emergence under field conditions

Treatments	Seedling emergence	
	Passo Fundo (2000)	Pato Branco (2001)
Untreated Check	247 c	238 c
Paenibacillus macerans(007)P	291 a	279 a
Paenibacillus macerans(007)WP	284 ab	270 a
Pantoea agglomerans(1494)P	290 a	268 a
Pantoea agglomerans(1494)WP	287 a	265 b
Pseudomonas fluorescens(4291)WP	279 b	267 b
Pseudomonas fluorescens(4291)P	278 b	264 b
Bacillus megaterium(9790)P	278 b	283 a
Bacillus megaterium(9790)WP	277 b	280 a
CV %	13.2	10.9

Table 3. Effect of seed-applied bioagents on wheat grain yield under field conditions, Pato Branco, PR, Brazil

Treatments	Yield (Kg/ha)	
	Passo Fundo (2000)	Pato Branco (2001)
Untreated Check	2135 c	2873 c
Paenibacillus macerans(007)P	2422 a	3182 a
Paenibacillus macerans(007)WP	2401 a	3173 a
Pantoea agglomerans(1494)P	3363 a	3135 a
Pantoea agglomerans(1494)WP	2307 a	3109 a
Pseudomonas fluorescens(4291)WP	2300 a	3104 a
Pseudomonas fluorescens(4291)P	2272 b	3088 ab
Bacillus megaterium(9790)P	2260 b	3180 a
Bacillus megaterium(9790)WP	2259 b	3174 a
CV %	9.7	11.6

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Effect of different isolates of *Trichoderma harzianum* on tomato pathogens

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Abstract: Ten isolates of *Trichoderma harzianum* were tested for their abilities to inhibit *Fusarium oxysporum, F. solani* and *Rhizoctonia solani*. All isolates inhibited the growth of pathogens on potato-dextrose agar; however, there were differences in biotic effect values between isolates. The isolates differed also in their protective effect in the pot experiment. The best results were obtained for *T. harzianum* isolate T 7, but the isolates T 21 and T 52 were also effective in the protection of tomato transplants against pathogens. Three isolates showed week antagonistic abilities to *Fusarium* spp. and four to *R. solani*.

Key words: *Trichoderma harzianum*, tomato, biological control

Introduction

Trichoderma species are known antagonists but their populations consist of strains with different antagonistic abilities (Henis et al., 1979). Their protective effect against pathogens depends not only on soil physical factors but also on the abilities of individual *Trichoderma* strains and the competitive properties of pathogens. Previous investigations proved that some fungal communities from the rhizosphere of tomato grown with cover crop could suppress the growth of *Fusarium* spp. (not published), thus testing of several *T. harzianum* isolates for their protective effect on some pathogens of tomato could present a fragment of antagonist population activity.

Materials and methods

T. harzianum isolates were obtained from the rhizosphere of tomato grown in field pea mulch. The isolates of pathogens were selected for their highest pathogenicity to tomato seedlings in previous pot tests. The antagonistic ability of *T. harzianum* isolates was tested in two experiments: dual Petri dish cultures and pot experiment.

The effect of *T. harzianum* isolates on the growth of selected pathogens on potato-dextrose agar was investigated using the biotic series method (Mańka, 1974; Mańka & Mańka, 1992). The transplants of tomato cv. Roma were planted into sphagnum peat with gravelite inoculated with pathogens (wheat grain grown through with mycelium). The plants were grown in growth chamber at 22-23 °C. *T. harzianum* was introduced as spore and mycelium liquid suspension (Czapek-Dox medium). For each pathogen there were two control combinations: peat without amendments and peat with pathogen inoculum. After 4 weeks the percentage of affected plants was estimated.

Results and discussion

All isolates of *T. harzianum* proved to be inhibitory to the growth of tested pathogens. However, the lower values of biotic effect were noticed in the test with *R. solani* what suggests that this fungus is the least susceptible to the activity of antagonists what contradicts some information (Geeta Sharma & Saxena, 2001). The differences in biotic effect values were also observed between *Trichoderma* species tested with individual pathogens. Some isolates (T 7, T 21, T 52) grew over pathogen colonies very quickly (+8) and other inhibited their growth not so strongly (Table 1). The results confirmed the data obtained in earlier investigations of *Trichoderma* spp. from the rhizosphere of wheat and field bean (Wagner, 1995, 1996).

Table 1. Biotic effect of *Trichoderma harzianum* isolates on the growth of tomato pathogens

Biotic effect on	Number of isolate									
	T 7	T 21	T 52	T 17	T 33	T 49	T 13	T 22	T 51	T 39
F. oxysporum	+8	+8	+8	+7	+7	+7	+7	+6	+6	+6
F. solani	+8	+8	+7	+7	+7	+7	+7	+6	+5	+6
R. solani	+8	+7	+7	+6	+6	+6	+5	+6	+5	+5

The differences in influence of *T. harzianum* isolates on health status of tomato plants have even been more pronounced. No isolate provided a complete control of pathogens. However, three isolates (T 7, T 21 and T 52) reduced significantly the percentage of affected plants and they can be regarded as promising biocontrol agents. Three isolates (T 22, T 51 and T 39) had poor antagonistic abilities against all tested pathogens. Moreover, the isolate T 13 reducing the number of diseased transplants by 75-80% in the combinations with *Fusarium* spp. was not so effective against *R. solani*. Generally, most of tested *Trichoderma* isolates showed lower inhibitory abilities to *R. solani* than to *Fusarium* spp. (Table 2).

That can be explained by several factors. *R. solani* is fairly resistant to antibiosis not only due to antibiotics production and specific hyphae morphology (thick walls, pigmentation) but also because of its preference of infection site. The pathogen attacks mostly at the ground level and grown downwards into roots, thus escaping *Trichoderma* (Kommedahl & Windels, 1979). The tested isolate was stem oriented as the first symptoms occurred on stem base of transplants. Also, *Trichoderma* species are the most effective in substrates with low pH and soluble phosphates deficiency (Henis et al., 1979). Even if the pH of substrate in pot experiment was unfavorable for the antagonist, three isolates showed high protective abilities against all tomato pathogens, what suggests their usefulness as biocontrol agents.

Table 2. Percentage of tomato plants infected with pathogens in pot experiment

Combination	*F. oxysporum*	*F. solani*	*R. solani*
Peat - no amendments	0	0	0
Peat + pathogen	100	100	100
T 7	5	13	10
T 21	10	10	10
T 52	14	13	25
T 17	25	20	30
T 33	25	13	30
T 49	20	20	25
T 13	20	25	45
T 22	40	35	40
T 51	40	45	55
T 39	45	45	55

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Evaluation of *Trichoderma* isolates for biocontrol of *Cercospora beticola* in sugar beet

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Abstract: Cercospora leaf spot, caused by *Cercospora beticola* Sacc. is one of the main sugar beet diseases in southern Europe, responsible for considerable reduction in root yield and sucrose content. The disease is generally more or less controlled through the use of tolerant genotypes and repeated fungicide applications. Recently, a great interest has arisen for alternative ways of control, due to the increasing demand for organic sugar and to the awareness for a safer environment. *Trichoderma* spp. are known as effective biocontrol agents towards a number of plant pathogens. This study reports the evaluation of some *Trichoderma* isolates as possible biocontrol agents of *C. beticola*. Thirty-five *Trichoderma* isolates of various origins, mostly from sugar beet soil, were tested *in vitro* for inhibiting *C. beticola* growth and also for *in vivo* spore survival on sugar beet phylloplane. Eight isolates with the best values were selected for a preliminary field trial in 2001, under natural *C. beticola* inoculum. Treatments did not affected the disease incidence, but three isolates significantly reduced *C. beticola* sporulation per unit of necrotic area in a way similar to the fungicide. Some of them gave also interesting sucrose yields, encouraging further studies.

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

Introduction

Cercospora leaf spot, caused by *Cercospora beticola* Sacc., is one of the most common and destructive diseases of sugar beet in southern Europe, responsible for considerable reduction in root yield and sucrose content. The disease control is generally achieved through the use of tolerant genotypes and repeated fungicide applications. Recently, a great interest has arisen for alternative ways of control, due not only to a greater attention to the environment but also to an increasing demand for organic sugar. *Trichoderma* spp. are known as effective biocontrol agents towards a number of pathogens, due to different mechanisms of action, such as competition, antibiosis and mycoparasitism (Tronsmo & Hjeljord, 1998). Even if the effectiveness of *Trichoderma* is widely demonstrated for different soil-borne pathogens, some positive results have been reported for aerial apparatus diseases, like that caused by *Botrytis cinerea*, for instance (Elad et al., 1993). The observation that both *Trichoderma* and *Cercospora* are necrotrophic fungi suggested the idea that they could compete at least for this aspect. This study reports the evaluation of some *Trichoderma* isolates as possible biocontrol agents towards Cercospora leaf spot.

Materials and methods

Trichoderma in vitro *screening*

Thirty-five *Trichoderma* isolates of various origins, mostly from sugar beet soil, were tested for antagonism towards *C. beticola*, measured as colony growth inhibition on Potato Dextrose Agar (PDA) amended with 25% (v/v) culture filtrate from each *Trichoderma* isolate. The filtrates were prepared by growing *Trichoderma* in flasks containing 50 ml Potato Dextrose Broth and incubated at 28°C for one week in the dark. The liquid cultures were then filtered through sterile cheesecloth and centrifuged at 3000 rpm for 20'. The sumatants were treated twice at 60°C for 30', to inactivate eventually remaining spores, and then added to PDA at 25% (v/v). Maximum diameter of *C. beticola* colonies was recorded after 15-day growth at 28°C, compared to that of control on PDA.

Trichoderma *spore survival test*

Trichoderma spore suspensions in 0,1% Tween 40 at the concentration of 1 x 10^6 spores/ml were sprayed on sugar beet leaves in greenhouse. After two weeks leaf sample of 1g were soaked in 50 ml water adding two drops of Tween 40 and shaken for two hours at room temperature. Then 0.5 ml were distributed on *Trichoderma* selective medium (Elad et al., 1981) in 9 cm Petri dishes and incubated in the dark at room temperature for two days. The Colony Forming Units (CFU) mg^{-1} of leaf were counted.

Sugar beet field experiment

Eight isolates with the best inhibition and spore survival values were selected for a preliminary field experiment on sugar beet, in 2001, under natural inoculum conditions in Rovigo, northern Italy (Table 1). Except for OR 6/99, which was isolated from strawberry soil, the remaining isolates originated from sugar beet soil. Sugar beet (cv Dorotea) plots of nine m^2, in a randomized block design with two replicates, were treated with *Trichoderma* spore suspensions in water (1L) at the concentration of 1 x 10^6 spores/ml every 20 days for five times, starting from the beginning of June until August 24th, when plots were scored for infection rate (0-9 KWS scale). The controls were water alone and ALTO BS (3% Cyproconazole and 9% Fentin acetate), sprayed on June 21st and July 10th at 2 kg/ha.

C. beticola *sporulation field test*

On September 4th, 10 leaves (5 x 2 blocks) for each treatments were randomly sampled, 10 disks with one spot each were cut from each leaf, put in a micro-centrifuge tube in 300 µl water and vortexed for 2', to release spores. The leaf disks were then computer scanned to calculate the necrotic area by the software program ImageJ 1.23 (http://rsb.info.nih.gov/ij/). Spore concentration was obtained as the mean of eight counts at Burker haemocytometer and the spore number mm^{-2} of necrotic area for each leaf was calculated.

Statistical methods

Data were submitted to analysis of variance. Percentages were arcsin transformed before analysis. Means were separated by l.s.d. test at $P \leq 0.05$ significance level, by MSTATC 2.1 microcomputer statistical program.

Results and discussion

Six out of the 35 *Trichoderma* isolates significantly differed from the control in inhibiting *C. beticola in vitro* growth and some showed also a good survival on sugar beet leaves. In addition

to the best six isolates for growth inhibition, we chose also 3 B 8 and ISCI 86/6, the first one being not statistically different from the other isolates in inhibiting *C. beticola* growth, the second one having a very good spore recovery value. Table 1 shows the values for these two characters for the eight isolates selected for the field experiment.

Table 1. *Trichoderma* isolates selected for the field experiment, inhibition of *C. beticola* growth on amended PDA, measured as colony diameter, and survival of *Trichoderma* spores, measured as Colony Forming Units (CFU) per mg of sugar beet leaf

Trichoderma isolates and species		*C. beticola* colony diameter (% of control)		*Trichoderma* spore recovery (CFU mg^{-1} of leaf)	
LT 2/99	*T. sp.*	78.8	g	10.0	a
LT 1/99	*T. sp.*	80.3	fg	1.9	cd
BA 12/86	*T. longibrachiatum* Rifai	82.6	efg	10.0	a
3 B 24	*T. viride* Pers.:Fr.	83.3	defg	2.2	cd
LT 5/99	*T. sp.*	83.3	defg	4.3	abcd
OR 6/99	*T. sp.*	85.6	cdefg	3.0	bcd
3 B 8	*T. koningii* Oudem.	89.4	abcdefg	3.4	abcd
ISCI 86/6	*T. sp.*	90.2	abcdef	10.0	a
Control		100.0	ab	0.0	d

Values followed by the same letter did not differ significantly (L.S.D. test, $P \leq 0.05$).

In the field, *Trichoderma* applications did not affected the disease incidence, which instead was well controlled by the fungicide (data not shown). The lack of effect was not due to a scarce persistence of the antagonist on the leaves. In fact, the survival of *Trichoderma* on the phylloplane was randomly checked 20 days after each application and spores were always found, despite sometimes heavy rains occurred. It is likely that *Trichoderma* did not affected *C. beticola* conidia germination, which, as it is known, rapidly occurs when free moisture covers the leaf surface. Further studies are in progress to elucidate this aspect, through the observation of the germination of *C. beticola* and *Trichoderma* spores in laboratory experiments.

Trichoderma applications seemed to be more effective in the subsequent saprophytic phase of the disease, when necrotic spots had appeared on the leaf surface. In fact, sampling the leaves at the end of the crop cycle, i. e. at the beginning of September, three isolates, BA 12/86, LT 1/99 and ISCI 86/6, were found to significantly reduce *C. beticola* sporulation per unit of necrotic area, compared to the untreated control (Figure 1).

It is interesting to note that their effect did not statistically differ from that of the fungicide, while the untreated control showed an almost double value of sporulation (Figure 1). These findings seem to confirm the hypothesis that there could be some competition between the two organisms for the colonisation of the necrotic spots, confirming also under field conditions what widely reported for other necrotrophic pathogens in controlled environment (Köhl & Fokkema,

1998). This aspect, once confirmed, could be exploited in integrated approach to control *C. beticola*, restricting the application of fungicide in the first phase of the disease and distributing *Trichoderma* based formulations in the later phases, reducing the sources of secondary inoculum.

Two of the best isolates for sporulation reduction, BA 12/86 and ISCI 86/6, gave also interesting sucrose yield, with absolute values higher than that of the untreated control, even if not statistically significant, probably due to the low number of field replicates (data not shown). Nevertheless these findings encourage further studies that are in progress.

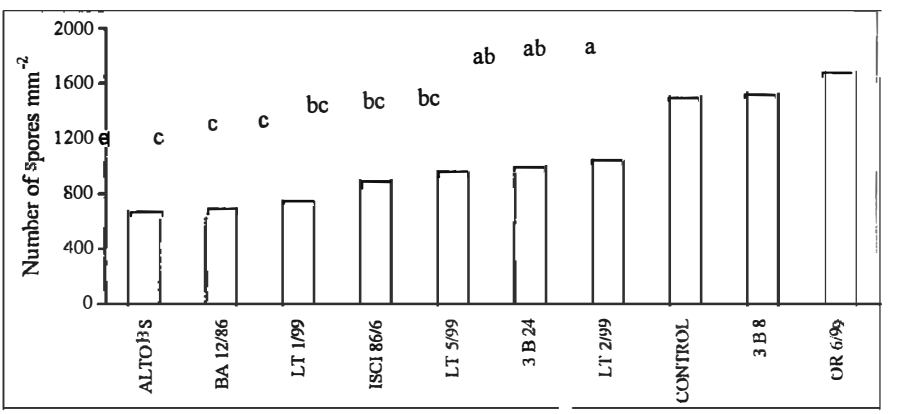

Figure 1. *C. beticola* sporulation from necrotic spots of sugar beet leaves in field after repeated *Trichoderma* applications. Bars with the same letter did not differ significantly (L.S.D. test, $P \square 0.05$).

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Isolation of fluorescent *Pseudomonas* and *Bacillus* spp. from the rhizosphere of pepper plants growing in suppressive soil and *in vitro* screening for antagonism against *Sclerotinia sclerotiorum*

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Abstract: A total of seventy-two strains of miscellaneous bacteria were isolated from the roots and crown of pepper plants growing in suppressive and non-suppressive soil to devastating root rot disease caused by *Sclerotinia sclerotiorum* in the different regions of Hatay. The antagonistic activities of these bacterial strains against fungal pathogen were screened *in vitro* in dual culture test. Among the inhibitory strains, six individual bacterial strains isolated from suppressive soil in different regions were selected for further evaluation. All six strains, consisted of three fluorescent *Pseudomonas* and three *Bacillus* spp., produced inhibition zones by inhibiting the radial growth of *S. sclerotiorum* to a varying degree. Strains of *Bacillus* were the most effective at inhibiting mycelial growth of fungal pathogen. Fungal growth was inhibited by 58% (SE=2.13) in the presence of the most efficient *Bacillus* strain. The extent of hyphal growth inhibition was influenced by pre-incubation time of bacterial inoculation to culture. Inhibition zone, produced by the most efficient *Bacillus* strain, increased proportionally with the pre-incubation time of bacterial inoculation prior inoculation with fungal pathogen in dual culture. Complete fungal growth inhibition was observed on PDA dishes in which antagonistic bacterial strain was streaked 4 days prior to inoculating the fungal pathogen. In addition to *in vitro* antagonistic activity of these bacterial strains, microscopical studies were made to reveal possible alterations on the fungal mycelium. The most efficient strain of *Bacillus* also caused cell wall lysis of fungal mycelium adjacent to the clear zone of inhibition. When the lysed mycelium was transferred on to PDA, fungus failed to grow on PDA. The effect of antagonistic bacterial strains on the viability of fungal sclerotia was also investigated *in vitro* by dipping sclerotia into different concentrations of bacterial suspension. Almost all bacterial strains had significant antagonistic effect on the sclerotia viability.

Key words: Fluorescent *Pseudomonas*, *Bacillus*, *Sclerotinia*, biological control, antagonism

Introduction

Sclerotinia stem and root rot (syn. white mold) caused by *S. sclerotiorum* has been considered an important seedling disease of pepper (Willetts & Wong, 1980). The pathogen survives in soils for several years by mass of specialised fungal survival structures such as sclerotia. For disease management, several strategies have been applied against the soil-borne pathogens to reduce the survival of the fungal resting structures. The high cost of pesticides, development of fungicides resistant pathogen isolates, governmental restriction on the use of fumigants, interest of environmental considerations, and the difficulty in finding suitable rotation crops to reduce pathogen inoculum, however, raise the need to find alternative control methods (Köhl et al., 1995). Biocontrol agents (BCA) such as fungi and bacteria are attractive and environmentally

friendly and feasible alternative to the chemical control and have been investigated intensively for several host-pathogen interactions (Weller, 1988). Rhizosphere of plant, growing in suppressive soil, contains several saprophytic bacteria which proved to be effective BCA against soilborne root pathogens of many crop plants (Larkin et al., 1996; Landa et al., 1997; Siddiqui et al., 2001). Suppression of soilborne pathogens in the field is mediated by plant growth promoting rhizobacteria (PGPR) belonging to the genera of *Pseudomonas* and *Bacillus*. Antibiosis, siderophore production, competition for substrate and niche exclusion and induction of disease resistance are well accepted mechanisms involved in disease suppression (Leong, 1986; Fravel et al., 1988; Wei et al., 1991). Isolation of bacterial isolates directly from suppressive soils could be a good alternative for the selection of BCA since they are already well adapted to survive in the field. The objectives of this study were to isolate bacteria from the rhizosphere of pepper plants growing in suppressive and non-suppressive soil to soilborne pathogens and to evaluate their *in vitro* antagonistic potential against *S. sclerotiorum*.

Materials and methods

Putative bacterial antagonists were isolated from the rhizosphere of pepper plants growing in suppressive and non-suppressive soil against soilborne pathogens in different region of Hatay during spring and summer period of 2001. Plant materials, included crown and roots, were extracted and spread onto the surface of King's Medium B agar (KB, Pseudomonas Agar F Base, Merck), which is selective for fluorescent pseudomonads, and Nutrient Agar (NA, Merck). Plates were incubated for 48 hr at 27 °C. All bacterial isolates were characterized according to Bergey's Manual of Systematic Bacteriology (Palleroni, 1984). The fungal isolate S. *sclerotiorum* used in this study was isolated from surface disinfected diseased pepper plants exhibiting symptoms of Sclerotinia rot and also from the sclerotia produced on stem. Isolates to be tested as potential antagonists were selected randomly from the single colony present on agar plates such that representative organisms from all colony and morphological types present were collected in approximate proportion to their abundance on the plates. Organisms other than fluorescent *Pseudomonas* and *Bacillus* were not identified but were assigned isolate number. Presumptive antagonistic bacterial isolates were screened on PDA and KB media for their ability to inhibit *S. sclerotiorum* in dual Petri dish culture test as described by Landa et al. (1997). Among the antagonistic bacteria, six selected bacterial isolates were further assessed for their inhibitory effect on hyphal growth and sclerotial germination as described before (Hoynes et al., 1999).

Results and discussion

Using the combination of techniques for the isolation procedures described above, a total of 72 bacterial isolates were obtained from the rhizosphere of pepper plants growing in suppressive and non-suppressive soils from 5 different regions. All collected isolates were screened individually for their ability to suppress fungal growth in dual culture test. From this test, 32 bacterial isolates (44.4%) were found to produce inhibition zones by inhibiting the hyphal growth of fungal pathogen to a varying degree. Among the antagonistic bacterial isolates, twenty two isolates (68.8%) originated from 3 different suppressive fields that were developed under wheat monoculture (disease incidences were less than 10%), four isolates from *Sclerotinia*, *Phytophthora* and *Fusarium* diseased open field (disease incidence was 35%), 3 isolates from

Sclerotinia and *Fusarium* diseased greenhouse (disease incidence was 85%) and 3 isolates from *Sclerotinia* diseased greenhouse (disease incidence 43%).

Isolates of antagonistic bacteria from suppressive soils were responsible for a large number of effective isolates (59.4%) than those from non-suppressive soil (6.2%). In suppressive soils disease suppression has been attributed to activity of specific non-pathogenic fungal and bacterial antagonists such as *Fusarium oxysporum*, and fluorescent *Pseudomonas* (Larkin et al., 1996). On the basis of the result obtained from the preliminary screening, the six bacterial isolates, obtained from different suppressive soils, found to be highly efficient against fungal pathogens. Three isolates (FP6, FP9 and FP16) were motile, gram negative, produced diffusible fluorescent pigment on KB agar, thus identified as fluorescent *Pseudomonas* sp. Fungal growth inhibition in PDA plates by these isolates was 48%, 31% and 35%, respectively. Several species of the fluorescent *Pseudomonas* spp., such as *P. fluorescence* and *P. aeruginosa* isolated from the rhizosphere of several plant species, have demonstrated antagonistic activity against sclerotial pathogens such as *Rhizoctonia solani*, *Macrophomina phaseolina*, *Sclerotinia rolfsii* (Siddiqui et al., 2001). Among attributed mechanisms, siderophore production has been proposed to be one of the efficient in disease suppression (Leong, 1986). We observed inhibition zone in dual culture test on PDA medium, which is iron rich media. Thus, not only siderophore production but also other mechanism(s) may be involved in the inhibition of hyphal growth of *Sclerotinia*. Similar result was also reported in the biocontrol of *Pythium* on cucumber plant (Paulitz & Loper, 1991). In a number of cases antagonistic effect of fluorescent *Pseudomonas* has been attributed to production of secondary metabolites such as antibiotics, salicylic acid and HCN (Weller, 1988). Other three isolates, namely B2, B3 and B11, were endospore-forming (subterminal position), gram positive, motile, oxidase and catalase positive. The solitary crinkled colonies of these isolates on NA were opaque, dull, and circular with an irregular circumference shape. No diffusible pigment was produced on KB Agar. Results suggest that these isolates belong to genus of *Bacillus*. The extent of hyphal growth inhibition was significantly ($P<0.01$) influenced by bacterial isolates tested. Isolate of *Bacillus* B3, showed the strongest antagonistic activity among all bacterial isolates tested. This isolate suppressed the fungal growth by 58%. Other two isolates (B2 and B11) inhibited the fungal growth on PDA by 43% and 48% respectively. Rhizosphere-inhabiting bacteria, belonging to genus of *Bacillus*, have been also shown to play an important role in the biocontrol of soilborne fungal pathogens (Yoshida et al., 2001). In the case of *Bacillus* isolate B11 deterioration of fungal mycelium adjacent to the clear zone of inhibition was observed 10 days of incubation. When deteriorated mycelial block was placed onto PDA plate, fungus failed to grow on PDA plate. Inhibition zone observed with *Bacillus* isolate increased proportionally with the preincubation time of bacteria prior inoculation with the fungal pathogen. Complete fungal growth inhibition was observed on PDA plate in which bacterial isolate was streaked 4 days before fungal inoculation. Production of antibiotic by different species of *Pseudomonas* and *Bacillus* in the late logarithmic or early stationary phase of growth in batch culture has been previously reported (Yoshida et al., 2001). In another approach to reduce the pathogen development, bacterial isolates were applied to sclerotia. The influence of each isolate on the viability of sclerotia of the fungal isolate was evaluated after 14 days of incubation in soil. Results revealed that almost all bacterial isolates had significant effect on sclerotial viability. The use of biocontrol agents in combination with fertilization or fumigation to reduced sclerotial viability of *S. rolfsii* has been investigated (Hoynes et al., 1999).

The results of our study conclude that fluorescent *Pseudomonas* and *Bacillus* spp. are the dominant antagonists and the primary organisms responsible for suppression of *Sclerotinia* and

probably other soilborne fungal pathogens in suppressive pepper soils, although other organisms may contribute to suppression. Isolation of bacterial strains directly from the suppressive soils could be a good alternative for the selection of BCA, since they are already well adapted to survive in the field. Selected isolates showing strong fungal antagonism, such as FP6 and B3, are promising for future applications of these bacterial isolates in biocontrol of soilborne pathogens. Currently, research is continuing to develop these antagonists as biocontrol agents with further investigations into their mechanism(s) of action in disease suppression. Subsequent analytical studies would lead to identification of compounds involved in disease suppression *in vitro* studies.

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Use of a microcosm system for biological screening against *Botrytis cinerea* in *Pinus sylvestris* seedlings

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Abstract: *Botrytis cinerea* (grey mould) is the most common and serious pathogen of economic significance in Swedish forest nursery production. Several sources of stress can predispose conifer seedlings to infection. In this work we present a microcosm bioassay, that includes a *Pinus sylvestris* seedling, and that will be used as one screening method to evaluate microorganisms for their antagonistic activity against *B. cinerea* in conifer seedlings. By observing the first infection events, we demonstrated that predisposition of 4 weeks old *P. sylvestris* seedlings is required for the establishment of a successfull *B. cinerea* infection. Scanning electron micrographs showed that most conidia of *B. cinerea* failed to germinate on the needle surface of non-predisposed seedlings at 3 days post inoculation (dpi) with 10^6 sp/ml in the microcosm bioassay. However, when seedlings were predisposed, germ tube formation and elongation was noticed already at 1 dpi, while needle surface colonization by hyphae, stomatal penetrations and appressoria were readily observed at 3 dpi.

Key words: antagonism, biological control, *Botrytis cinerea*, forest nursery, *Pinus sylvestris*, screening, predisposition

Introduction

Grey mould (*Botrytis cinerea*) is a widespread and frequently encountered disease problem of economical importance at all stages of seedling production in forest nurseries (Mittal et al., 1987). Of all fungal diseases in Swedish forest nurseries, *B. cinerea* is the most common both in bare root and specially in container-grown seedlings (Nyström et al., 2001). Sweden produces about 300 million seedlings yearly and about 80% of all seedling production is grown in containers.

Generally *B. cinerea* is a saprophyte and initial symptoms are usually found on the lower parts of seedlings in dense canopies during late summer. *B. cinerea* can become severe during autumn, cold storage and transport for outplanting. Several sources of stress can predispose seedlings to infection (Schoeneweiss, 1981) and unfortunately, nursery practices cannot prevent to stress seedlings. Zhang et al. (1995) showed that reduced photosynthesis associated with low light intensity in dense planting, high temperature and drought predisposed black spruce (*Picea mariana*) seedlings to infection by *B. cinerea* (Zhang et al., 1995). Other potential stress factors involve abrupt chilling or freezing, long humid periods and seedling nutrition state. Factors favouring grey mould include high relative humidity or availability of free water, temperate temperature, senescing of needles and presence of exogenous nutrients stimulating spore germination on the plant surface (Elad, 1996; Mittal et al., 1987).

Most Swedish forest nurseries deploy mainly routinely applied, fungicidal sprayings to try to prevent fungal infections. However, only two chemical active ingredients (iprodion and tolylfluanid) are currently registered for use against *B. cinerea* in Swedish forest nurseries.

Furthermore, to diminish human health risks, environmental concerns and development of resistance to fungicides, alternative methods such as biocontrol, are highly needed.

In this study a microcosm bioassay was developed, that will be used later on for screening of microorganisms for biocontrol activity against *B. cinerea* in conifer seedlings. We also studied the first infection events of the pathogen on the needle surface by scanning electron microscopy.

Material and methods

Microcosm bio-assay

Seeds of *Pinus sylvestris* were surface sterilized in 33% H_2O_2 and rinsed several times with sterile water before sowing on water agar. One week later, pine germlings were transferred to Eppendorf tubes containing Ingestad nutrient solution (Sharma et al., 1993). Germlings were then grown for another 2 weeks at room temperature and 16 h photoperiod. Prior to inoculation of the foliage, the seedlings were subjected to warming to 40°C. Control seedlings were kept at room temperature. The inoculation solution consisted of water with 0.05% Triton X-100 and 10^6 sp/ml *B. cinerea*. To recover conidia, pure *B. cinerea* cultures were flooded with 0.05% Triton X-100 in water and the spore suspension was filtered through a fine mesh. Spore concentration was estimated by aid of a haemocytometer. Inoculated seedlings were then transferred to a testtube containing water at the bottom to ensure high relative humidity and silikon plugs to close off the test tube. Incidence of conidiophores and sporulation of *B. cinerea* on the needles was recorded from seven to ten days post inoculation (dpi).

Scanning electron microscopy

Needle segments of seedlings that were predisposed, were fixed at 1 and 3 dpi in 2.5 % glutaraldehyde and PBS buffer at 4 °C, while needle segments of control plants were fixed at 3 dpi only. After 2 days, tissues were rinsed in the same buffer and post fixed in 1% OsO_4 at room temperature during 2 h. After rinsing the tissues in buffer several times, samples were dehydrated in a graded ethanol series to 100% EtOH. Needle segments were then critical point dried, mounted on stubs and sputter coated. Specimens were then observed with a Philips scanning electron microscope at 20 kV.

Results and discussion

Many screening programs in biocontrol of plant pathogens are based on dual culture interactions on agar media in Petri dishes, but ecological relevance of these tests are open to doubt. Although they can reveal the involvement of potential important modes of action such as antibiosis and competition for space and nutrients, several other mechanisms like induced resistance, niche exclusion, colonization and competitive ability on the plant surface are not involved in such *in vitro* tests. Therefore we developed and standardized a microcosm bioassay, as described above, that will allow us to evaluate microorganisms for their biocontrol activity against *B. cinerea* in *P. sylvestris* seedlings.

Before initiation of screening, parameters in the microcosm bioassay were optimized for successful establishment of *B. cinerea* infections in *P. sylvestris* seedlings. The first step for a pathogen prior to invasion of plant tissue is the adhesion ability of spores to the plant surface. Scanning electron microscopy showed that conidia were able to attach to the needle surface, usually on the anticlinal walls between epidermis cells, when seedling foliage was dipped into a solution containing pathogen spores. It seemed that seedling predisposition by heating prior to inoculation is an important step in the microcosm bioassay in order for the spores to germinate. Scanning electron micrographs showed that most of the conidia on the needle

surface of non-predisposed plants failed to germinate three days post inoculation. However, when seedlings were predisposed, germination of conidia and germ tube elongation were noticed already at one day post inoculation, while hyphal growth and needle colonization were obvious at three days post inoculation. At this latter time point, hyphal tips showed taxis towards but sometimes also seemed to avoid epistomatal openings. We observed that penetration of needles occurred frequently through stomatal openings. Appressoria were relatively common and lead to direct penetration through the cuticle. Similar observations on infection events were done by Dugan & Blake (1989) on older western larch (*Larix occidentalis*) seedlings. At ten days post inoculation symptoms were observed under a stereo microscope: non-predisposed seedlings remained healthy and were comparable to control plants inoculated with water and surfactant only, while predisposed seedlings showed abundant sporulation of *B. cinerea*.

Based on these results, it seems that predisposition in the microcosm bioassay is crucial for a successful infection in *P. sylvestris* seedlings. Zhang & Sutton (1994) also showed that grey mould failed to develop in completely green black spruce (*Picea mariana*) seedlings, but not when seedlings were predisposed at high temperature and in darkness during four days. Since *B. cinerea* needs exogenous nutrients for germination (Elad, 1996), it is probable that predisposition at high temperature causes small cracks in plant tissue and leakage of nutrients from the damaged host tissue to the needle surface. It is also possible that predisposition interferes with plant defence mechanisms, thereby facilitating pathogen invasion. In Swedish forest nurseries, production of seedlings in containers can lead to predisposition associated with dense planting and low light intensity after canopy closure. Knowledge on predisposition factors can help forest nursery people to ameliorate nursery practices in order to stress less seedlings.

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Selecting fungal biocontrol agents amenable to production by liquid culture fermentation

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Abstract: Numerous fungi show excellent potential for use as biocontrol agents due to their ability to selectively infect and kill a variety of weedy and insect pests or their ability to antagonize or exclude plant disease-causing organisms through parasitism or competitive exclusion. The lack of suitable methods for economically producing stable, effective fungal propagules continues to impede the commercial use of these biocontrol agents. Selecting fungi for use as biocontrol agents and for concomitant amenability to liquid culture production requires an understanding of how the organism carries out its biocontrol function and in what environment it will be used. For use as a foliar spray or in post-harvest disease control applications, production of the fungal biocontrol agent as yeast or yeast-like propagules is advantageous. The potential to control soil-borne plant diseases with fungal biocontrol agents is greatly enhanced if the agent produces propagules such as chlamydospores or sclerotia that are capable of persisting in the soil environment. Our research focuses on developing media and processes for the liquid culture production of promising fungal biocontrol agents. To demonstrate how nutritional factors can be used to regulate propagule formation and propagule "fitness" during liquid culture production, results from our studies with the mycoherbicide *Colletotrichum truncatum* and the mycoinsecticide *Paecilomyces fumosoroseus* will be presented. High concentrations of desiccation-tolerant, yeast-like blastospores of the fungus *P. fumosoroseus* were produced in liquid culture when supplied medium containing an appropriate concentration and source of nitrogen. Nutritional studies with *C. truncatum* cultures grown in liquid media demonstrated that sporulation or microsclerotia formation was regulated by the carbon concentration of the medium. High concentrations of desiccation-tolerant microsclerotia of *C. truncatum* were produced in liquid media that contained a high carbon concentration. The potential of using microsclerotia as soil amendments for controlling soil-borne plant diseases will be discussed.

Key words: Biological control agents, fungi, nutritional environment, liquid culture production, fermentation, biopesticide, mycoherbicide, mycoinsecticide

Introduction

In 1987, Dr. Kenneth F. Baker documented a sharp increase in research activity on the biocontrol of plant pathogens. He noted that from 1960 to 1981, the number of papers published per year on the topic rose from approximately 15 to nearly 200. The publication rate of papers on the biocontrol of plant pathogens has been maintained and likely exceeded in subsequent years. However, though the disease controlling activity of literally hundreds if not thousands of microbial strains have been described in publications (Paulitz & Bélanger, 2001), only a small percentage of biocontrol strains have reached the stage of commercial development (Fravel & Larkin, 1996; Wraight et al., 2001).

Likewise, hundreds of fungi have been identified which are candidates for development as commercial bioherbicides and bioinsecticides. These fungi demonstrate host specificity and

can be highly aggressive in infecting and killing their insect or weed host. Despite this success in discovering potential biocontrol agents, only five fungal bioherbicides and three fungal bioinsecticides have been registered for use in the United States and Canada.

The overall lack of commercial success in using living microbial biocontrol agents stems from difficulties in producing and stabilizing these agents and from inconsistent pest control in field situations. Required are low-cost production methods for fungal biopesticidal that yield high concentrations of viable, effective propagules. The development of liquid culture fermentation processes that overcome these problems is necessary for the commercialization of many microbial biocontrol agents. A close look at the nutritional composition of the production medium is necessary since nutrition has been shown to have a dramatic impact on propagule attributes such as biocontrol efficacy and desiccation tolerance.

This presentation will outline strategies for selecting fungal biocontrol agents that are amenable to liquid culture production methods. Selection factors that will be considered include how the fungus grows and differentiates in liquid medium, the stability and biocontrol efficacy of the resulting propagules and how the agents will be used in practice as a biopesticide. To demonstrate this strategy, results from our nutritional studies with the fungus *Colletotrichum truncatum*, a specific pathogen of the weed hemp sesbania, and *Paecilomyces fumosoroseus*, a pathogen of various soft-bodied insects including the silverleaf whitefly, will be presented.

Materials and methods

Over the past 12 years, we have evaluated numerous fungi for amenability to liquid culture production. The general methods employed to examine these fungi are outlined in our liquid culture production studies with the fungal biocontrol agents *Colletotrichum truncatum* and *Paecilomyces fumosoroseus*. References to these experimental methods are outlined in the following manuscripts: Jackson, 1999; Jackson and Bothast, 1990; Jackson and Schisler, 1994; Jackson et al, 1997; Schisler et al, 1991. Fungal spores and microsclerotia obtained from these studies were evaluated for biocontrol efficacy, desiccation tolerance and stability as described in the following manuscripts: Cliquet & Jackson, 1999; Jackson et al., 1997; Schisler et al., 1991; Schisler & Jackson, 1996).

Results and discussion

The use of microbial biocontrol agents in commercial agriculture is limited by biological and economic factors. Ecological and environmental factors conspire to make consistent pest and disease control under field conditions difficult to achieve. Most agronomic crops can afford only modest monetary inputs for pest and disease control, particularly when the control agent is directed at one or a small number of pests or diseases. Selecting fungal biocontrol agents for commercial development must take these economic and biological factors into consideration. Likewise, evaluation of fungi for amenability to liquid culture production must consider how the agent will be used to control the target pest or disease. This information can then be used to determine if a suitable fungal form or propagule can be produced using submerged culture techniques. Our studies with fungal biocontrol agents have focused on developing liquid culture production methods that economically produce stable fungal propagules capable of providing consistent pest control under field conditions. By manipulating the nutritional environment present during fungal growth, we have developed schemes for screening fungi for their amenability to liquid culture production. In these

studies, fungi are evaluated based on their mode of growth and differentiation, propagule yield and stability, and biocontrol efficacy.

If the fungal biopesticide is to be applied as a spray (i.e. "contact" biopesticide), the production method employed must yield high numbers of discrete, infective propagules. Yeasts or dimorphic fungi that can be induced to grow in a "yeast-like" fashion in liquid culture are preferred candidates for this application since, for example, application rates for insect control can approach 2.5 X 10^{13} spores/hectare. Clearly, economic considerations dictate that the production method developed must rapidly produce very high yields of discrete, stable, infective propagules. Plant pathogens such as *Colletotrichum gloeosporioides* and numerous entomopathogens of the genera *Paecilomyces, Beauveria, Verticillium* and *Metharizium* can be induced to grow in yeast-like fashion in submerged culture. Our studies with the fungal bioinsecticide *Paecilomyces fumosoroseus* have demonstrated that "yeast-like" blastospores of this fungus can be rapidly produced in high concentrations if appropriate concentrations of nitrogen are provided (Jackson et al, 1997). The rapid germination rate of *P. fumosoroseus* blastospores makes these propagules ideal candidates for use as a contact biopesticide. For conidia production in liquid culture, a vegetative fungal growth cycle is required followed by hyphal differentiation to form conidia. This process requires added fermentation time and separation of conidia from mycelia for spray application (Table 1). The inefficiencies and added costs associated with the submerged conidiation process often preclude its use for most "contact" biopesticides.

Table 1. Fungal propagules produced in liquid culture

Propagule	Production Time	Stability	Biocontrol Efficacy
Vegetative hyphae	2-3 days	Poor	Fair
Conidia	4-6 days	Poor to Good	Good to Excellent
Microsclerotia	5-8 days	Excellent	Good to Excellent
Yeast-like	2-4 days	Poor to Good	Excellent

For fungal biocontrol agents that are to be used as seed coatings or as granular applications in soil or aquatic environments, a very stable fungal propagule is required. Conidia or "yeast-like" propagules are often not sufficiently stable or amenable to these applications. Conversely, fungal propagules such as microsclerotia or chlamydospores that often function as overwintering structures for many fungi are well suited to use as granular biopesticides. Our studies showed that when *C. truncatum* was grown in liquid media containing a high concentration of carbon, dense vegetative growth was followed by the formation of high concentrations of melanized, compact hyhal aggregates which we have termed "microsclerotia" (Jackson and Schisler, 1994). Microsclerotia of *C. truncatum* are extremely stable as dry preparations, germinate in hyphal or sporogenic fashion, infect and kill emerging hemp sesbania seedlings and remain viable when used as coatings on soybean (D. Boyette, unpublished data). Recent studies have shown that liquid fermentation can be used to produce microsclerotia of other fungi, including *Mycoleptodiscus terrestris*, a pathogen of the aquatic weed *Hydrilla verticillata* (J. Shearer, unpublished data). The further development of cost-effective, liquid culture production techniques for fungal microsclerotia has potential to heighten commercial interest in using granular fungal agents as seed coatings, soil amendments or in aquatic applications. Potential also exists for using these microsclerotial propagules as soil amendments to enhance populations of beneficial mycoparasitic fungi. If mycoparasitic fungi that infect and kill sclerotia of plant pathogenic

fungi such as *Sclerotinia* or *Rhizoctonia* sp. are capable of using liquid culture produced microsclerotia as a refuge or food source, it may be possible to create suppressive soils through augmentation with microsclerotia. The authors are currently conducting collaborative studies to test this hypothesis.

In conclusion, commercial biopesticide development requires selecting environments and biocontrol agents that meet strict economic and biological criteria. Biopesticide production methods must be low-cost and yield stable, effective agents that are suited to the application method used to control the pest or plant disease. Biopesticide production using liquid fermentation methods is best suited to fungi that grow "yeast-like" or produce microsclerotia. The nutritional environment present in the liquid culture medium can have a dramatic impact on the form, yield, stability and efficacy of the biopesticidal agent.

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Screening of plant extracts, micro-organisms and commercial preparations for biocontrol of *Phytophthora infestans* on detached potato leaves

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Abstract: Throughout the EU, late blight caused by *Phytophthora infestans* causes substantial economic losses in organic potato production systems. The proposed ban on copper fungicides will substantially increase economic losses of farmers, unless suitable alternative blight management strategies are developed. Within the EU funded project 'Development of a systems approach for the management of late blight in EU organic potato production' one important aspect is the development of alternative control treatments based on plant extracts and/or micro-organisms. An excised leaf bioassay was developed and the effect of altogether 122 micro-organisms (isolated from the phyllosphere of potatoes), plant extracts and commercial preparations (in the following collectively termed "test substances") on the development of *P. infestans* was evaluated. The mode of action (curative or protective) was taken in consideration by applying the test substances 24 hours before or one hour after inoculation with *P. infestans*. In general, protection was better when the test substances were applied before the pathogen. Some of the test substances were highly effective, even comparable to the copper treatment. When 23 effective micro-organisms (spore forming and non spore forming bacteria, yeasts and fungi) were tested in dual cultures, different patterns of inhibition of *P. infestans* were observed. A correlation between the inhibition in 'in vitro' assays and effectiveness in the leaf assays was not recorded. The results indicate that various modes of action can be expected. In an additional bioassay Serenade® was identified as the best biocontrol product.

Key words: *Phytophthora infestans*, biological control, *Solanum tuberosum*, potato, micro-organisms

Introduction

Late blight (caused by *Phytophthora infestans*) is one of the most important diseases affecting organic and conventional potato production world wide. Under suitable environmental conditions the disease can spread very rapidly and can cause complete crop losses. Protective copper fungicides, which are currently used to control the disease in most organic production systems, are estimated to extend the length of the growing season by between 10 to 30 days. However, a replacement of copper by alternative control treatments is desired. For the control of fungal pathogens various alternative treatments have been developed, including microbial antagonists and also plant extracts, which have an effect on the fungus by direct antifungal effects or stimulation of competitor micro-organisms and/or effects on the plant via resistance inducing "plant strengthening" activities. However, there are few reports of successful alternative control approaches using these treatments against late blight. Therefore, micro-organisms, isolated from the phyllosphere of potatoes, plant extracts and microbial preparations were screened for late blight control.

Material and methods

Production and processing of micro-organisms / plant extracts

Bacterial cultures were produced in nutrient broth (Oxoid). After incubation for seven days at 20° C and 180 rpm the bacterial culture was centrifuged and the pellet was resuspended with 0.0125% Tween 80. The centrifugation was repeated three times to wash out metabolites. The bacterial suspension was diluted to an optical density of 0.9 at 660 nm. Yeast cells were produced in a medium containing 3 % maltextract 0.5 % peptone. The suspension was adjusted to an optical density of 2.2 at 660 nm wavelength. Fungal micro-organisms were cultured on petri dishes containing maltextract-peptone agar for 14 days at 20° C. Fungal spores were washed from the petri dishes with 0.0125 % Tween 80 and a concentration of $1x10^6$ spores per ml was adjusted. *Phytophthora infestans* was cultured on petri dishes on rye agar + Sitosterol for 14 days at 15° C. Fungal sporangia were washed from the petri dishes with 0.0125 % Tween 80 and a concentration of $5x10^5$ spores per ml was adjusted. For the release of zoospores the suspension of sporangia was incubated for one hour at 5° C. Freshly harvested plant material was dried at 60° C and milled. Cold water plant extracts were prepared by extracting 0.5 g plant material in 50 ml 0.0125 % Tween 80. The extracts were prepared immediately before use. Products and experimental products were applied as a 1% suspension.

Application, incubation and assessment

Potatoes (var. Secura) were cultured for four weeks and leaves of the 5^{th} to 7^{th} branch were collected and placed onto steel wire mesh with soaked filter paper underneath in 20x20x5cm plastic boxes. A suspension of 2.5 ml of antagonists and/or plant extracts were applied 24 hours before or one hour after inoculation of the pathogen. For the application of the pathogen 2µl of the *P. infestans* suspension was applied at 1 cm distance from the leaf vein of the terminal leaf. Four leaves were applied per treatment. Treated leaves were incubated at 15°C. For classifying the infection five infection levels were defined. After seven days incubation the value of infection was determined. For analysis the percentage of effectiveness the data were calculated following the formula of Abbott (1925).

Dual culture of effective micro-organisms

Based on the first excised leaf assay 23 micro-organisms were selected for dual cultures. *P. infestans* was placed in the centre of the agar-plate and after three days incubation two stripes of the micro-organism culture were plated in a distance of two cm from the centre. After 11 days the width and length of *P. infestans* colony was measured and with regard to the untreated control the inhibition was calculated.

Screening of selected products and plant extracts

The most active plant extracts (*A. vulgaris, I. parviflora, R. rhabarbarum, S. canadensis, U. dioica*) and most active products (Elot-Vis, Serenade, TRICHODEX, T 1, T 2) were compared within an additional leaf assay. In contrast to the first assay the inoculation of *P. infestans* was performed by spraying a suspension of zoospores ($1*10^5$ sporangia/ml) on the excised leaves. For each of the three replications three boxes with four leaves were treated. The percentage leaf area covered was determined following James (1971). Means of the arcsine transformed data were compared by the Tukey's studentized range test ($P \leq 0.05$).

Results

In the first screening altogether 99 micro-organisms, nine plant extracts and 14 products were tested. Some substances in each group showed an effect on *Phytophthora infestans*. There was no correlation between curative or protective activity, but in general, the effectiveness

was higher when the test substances were applied 24 hours before *P. infestans* (Figure 1). When selected (effective) micro-organisms were tested in dual cultures, different patterns of inhibition of *P. infestans* were observed. A correlation between the inhibition in 'in vitro' assays and effectiveness in the leaf assays was not recorded (Figure 2). Eight of the selected isolates showed an inhibition of the width of over 50 %, 12 between 25 to 50% and only three isolates caused an inhibition of lower than 25%. When the length of the culture was measured, 5 isolates showed an inhibition of over 50%, nine isolates between 25-50% and nine isolates caused an inhibition lower than 25 %. No correlation between the inhibition within the *in vitro* assays and effectiveness within the leaf assays was visible.

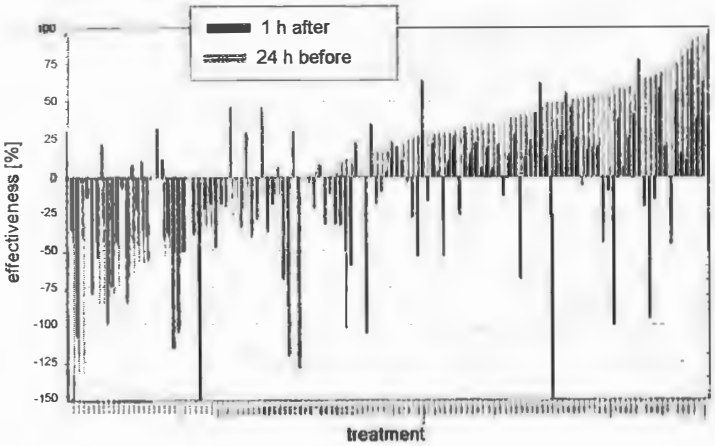

Figure 1. Effectiveness in leaf assays of 122 test substances

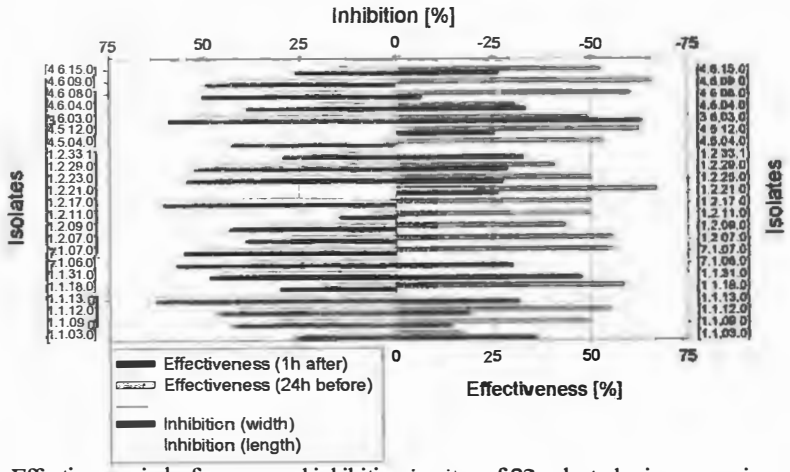

Figure 2. Effectiveness in leaf assays and inhibition *in vitro* of 23 selected micro-organisms

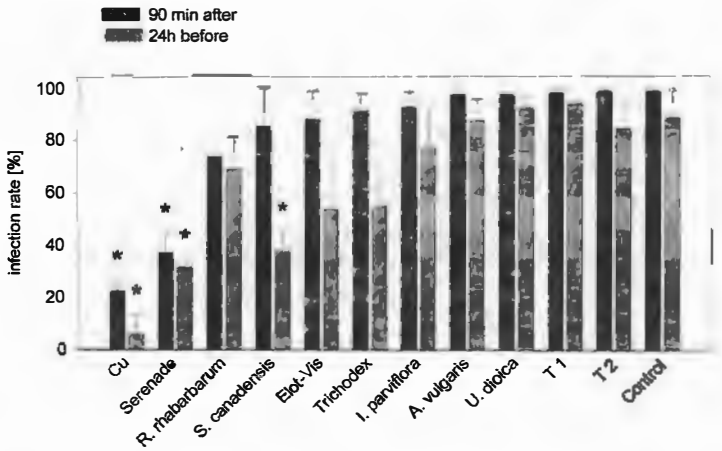

Figure 3. Inhibition rate of selected products and plant extracts of *P. infestans*

In leaf assays with spray inoculation of *P. infestans* the infection rate of the controls was 100%. When applied 24 hours before *P. infestans*, the infection rate was 23% in the copper treatment. With an infection rate of 38 %, Serenade® (*B. subtilis*) was the best alternative treatment and did not differ significantly from the copper control. In the assay with application of the test substance after the *P. infestans* inoculation, Serenade and *S. canadensis* caused an infection rate of 32 and 38 %, respectively, with no significant difference to the copper-control (Figure 3).

Conclusion

The results indicate that some of the tested micro-organisms have an effect on *P. infestans*. Serenade appears at present to be the most promising product. Greenhouse and field trials will be carried out.

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Ecophysiological manipulation of fermentation improves viability of the biocontrol yeast *Pichia anomala*

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Abstract: For effective biocontrol to be achieved it is important that cheap and economic substrates can be used to produce ecologically competent inocula. To this end we have examined and compared the production of the biocontrol yeast *P. anomala* on rich defined media (NYDB) and on molasses. Manipulation of the physiology of the yeast by modification of water stress [water activity (a_w) of 0.98 and 0.96] using different compatible solutes/sugars and NaCl markedly affected yield and quality of the cells. Endogenous water potential of cells, and sugar/sugar alcohol contents were significantly modified. In general, accumulation/synthesis of trehalose or sugar alcohols was affected by the solute used in media. Ecological competence of the yeast treatments was examined by plating on non-stressed (0.995 a_w) and water stressed media (0.96 a_w). Viability was significantly improved by the use of proline and NaCl in molasses media. Such studies have implications for improving shelf-life and perhaps the production of ecologically stable biocontrol agents.

Key words: biological control agents (BCAs), *Pichia naomala*, molasses, water activity (a_w), cell water potential (Ψ_c), compatible solutes, polyols, sugars, trehalose, improved viability

Introduction

The increasing public concern about environmental matters and the pressure for healthier food products combined with the ban or withdrawal of several pesticides and pathogen resistance to several chemicals worldwide, favour the use of fungal biocontrol agents (BCAs), both preharvest and postharvest, as an alternative method to chemical treatments (Butt et al., 2001). However, for BCAs to be successfully used, several key aspects of their development, including economic mass production, ecological fitness, formulation and shelf-life of the final product need to be optimised. Previous studies with BCAs mainly focused on fermentation optimisation so that high inocula production was ensured. Nevertheless, improved quality and ecological fitness of inoculum are of great importance as BCAs need to be able to withstand the natural fluctuations in humidity and temperature of their environmental niche.

Water activity (a_w) and water potential Ψ_w (Pa) are two thermodynamic parameters widely used in microbiology. The former describes the water availability for microbial activity (Magan, 1997) while the latter permits the partition of osmotic and matric components and thus describes their influence on growth and physiological functioning of microbes. Ψ_w is related directly to a_w by the following formula: $\Psi_w = RT/V_w \ln a_w$; where R is the ideal gas content, T the absolute temperature and V_w is the volume of 1 mole of water.

For fungal cells to function, grow and reproduce under water stress they need to adjust their internal Ψ_c to a level lower than that of the immediate environment Ψ_w. For this scope, yeast cells intracellularly accumulate the low molecular mass polyhydric alcohols (polyols) (glycerol, arabitol, erythritol and mannitol), which are compatible with metabolism and confirmation of proteins (Brown & Simpson, 1972). The disaccharide trehalose accumulates

in the cells in response to osmotic stress. It has been suggested that trehalose enhances desiccation tolerance of conidia of the BCA *T. harzianum* (Harman et al., 1991).

Björnberg & Schnürer (1993) first showed that *P. anomala* J121 strain (originally isolated in 1981 from airtight-stored grains) effectively reduced growth of *P. roqueforti* and *A. candidus in vitro*. The objective of this study was to investigate the impact that changes in solute stress have on yield, endogenous water potential (Ψ_c), quality and ecological competence of *P. anomala* cells when grown on rich defined media (NYDB) and on cheap industry byproducts such as molasses.

Materials and methods

Microorganism used

The microorganism used in this study was *Pichia anomala* (strain J121), originally isolated in 1981 from airtight-stored grains (Björnberg & Schnürer, 1993). Stock cultures were stored at 4 °C and subcultured on Malt Extract Agar (MEA, MERCK) as required.

Culture media used and their modification

Nutrient yeast extract broth (NYDB) made of 8 g/l nutrient broth (Oxoid Ltd.), 5 g/l yeast extract powder (Lab M, IDG Ltd.), 10 g/l D-(+)-glucose (Sigma-Aldrich, Ltd.); pH 6.6 ±0.2 and a_w 0.993-0.996. Cane molasses-based medium made of cane molasses 40gl^{-1} and urea 1.2gl^{-1}. Molasses was kindly supplied by UdL-IRTA, Lleida, Spain; pH 6.1/a_w 0.993-0.996. Modification of media a_w (0.98 and 0.96 levels) was made by the addition of the solutes glycerol, glucose, sorbitol, proline and NaCl as described by Abadias et al. (2000).

Yield Studies

Yield was measured using a regression line of Absorbance (700 nm) against dry weight.

Water potential measurements

Thermocouple psychrometry was used to determine the water potential of the cells. The procedure was described previously by Abadias et al. (2000).

Viability and ecological competence

Fifty ml of medium of each treatment (in 250 ml Erlenmeyer flasks) were inoculated and incubated on a rotatory shaker (150rpm) for 72h at 25 °C. Viability of *P.anomala* cells was assessed by spread-plating on unstressed (0.995 a_w) and stressed NYDA (0.96 a_w modified with polyethylene glycol 200, 1.25 m) media. Plates of the same a_w were sealed in polyethylene bags to prevent water loss and incubated at 25 °C. CFUs were counted after 48 h for unstressed and after 120h for water stressed NYDA media.

Extraction and quantification of intracellular polyols and sugars

Extraction/quantification of polyols/sugars was done according to Abadias et al. (2000).

Results

Yield of* P. anomala *in grown in NYDB and molasses liquid media

Figure 1 shows the effect of a_w on yield of *P. anomala* grown in NYDB and molasses liquid media after 72h incubation at 25°C. In general, yield decreased as media a_w decreased. Yield was dependent on the solute used to modify water stress. When glucose was used to modify medium to 0.98 a_w yield was stimulated. In NYDB media, glucose, followed by glycerol, gave the best results among the solutes tested. In molasses media, glucose, followed by glycerol and, interestingly, NaCl, gave the best results. In general, yield produced in NYDB media was higher than that produced in molasses media.

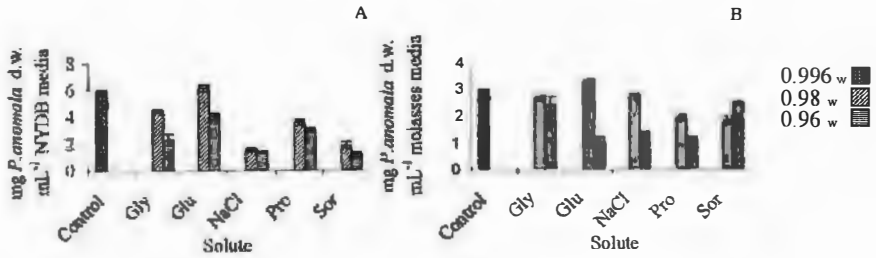

Figure 1. Yield of *P. anomala* cells grown in NYDB (A) and molasses (B) media after 72h of incubation at 25 °C

Water potential measurements of* P. anomala *cells

For each solute tested, a decrease in medium a_w resulted in a decrease in Ψ_c. Yeast cells grown in unmodified media had the highest Ψ_c values. In general, NaCl treatments gave the lowest Ψ_c values when media were modified at 0.98 a_w and 0.96 a_w. Glucose treatments gave the highest Ψ_c values especially in NYDB media (data not shown).

Viability and ecological competence of* P. anomala *cells grown in molasses media

Proline 0.96 a_w and NaCl 0.98 and 0.96 a_w treatments when plated on unstressed NYDA media gave the highest viable counts, higher than the unmodified molasses medium. On water stress medium viability was also high (>9.33×10^9 CFU/ml). In NaCl 0.98, Pro 0.96 and NaCl 0.96 a_w treatments yeast cells were particularly water stress tolerant (data not shown).

Intracellular sugar and polyol accumulation in* P. anomala *cells grown in molasses media

Figure 2 shows an example of the changes obtained in the polyols and trehalose content of *P. anomala* cells; proline was used to modify molasses media a_w. In the unmodified media trehalose was found to be the predominant intracellular compatible solute followed by arabitol. In proline/0.98 a_w treatment, trehalose was found to be strongly intracellarly accumulated (61.24 mg/g f.w.) followed by arabitol (31.85 mg/g f.w.). Cells from the proline 0.96 a_w treatment also accumulated high quantities of trehalose (48.36 mg/g f.w.) followed by arabitol (29.68 mg/g f.w.); however, glycerol was mostly synthesized (21.05 mg/g f.w.). In all treatments glucose accumulation was not found.

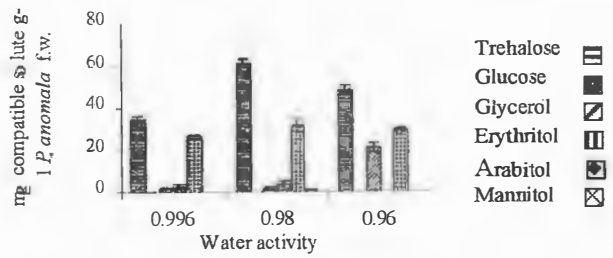

Figure 2. Endogenous concentration of sugars and sugar alcohols of *P. anomala* cells grown in Proline modified molasses media after 72h incubation at 25 °C

Discussion

This study is the first investigation of the impact that changes in solute stress have on yield of the yeast *P. anomala* when grown in both rich defined media (NYDB) and in cheap

byproducts from the sugar processing industry. Effects on endogenous sugar/polyol accumulation, viability of the cells in unstressed and water-stressed NYDA media and cell water potentials were also investigated. It has been shown that a_w and solute type have a significant effect on growth, Ψ_c, viability of cells and endogenous accumulation/synthesis of sugars and polyols. Moreover, yield and endogenous accumulation/synthesis of sugars and polyols were dependent on the substrate used.

In general, defined media (NYDB) gave higher yields than molasses-based media. A_w modifications caused a change of Ψ_c so that $\Psi_c = \Psi_w$, with cells from glucose modified NYDB media the only exception. These results support Magan (1997) who underlined the importance of the cells ability to be in osmotic equilibrium with the surrounding environment in order to prevent swelling of the cytoplasm. Studies by Abadias et al. (2000) on effects of medium Ψ_w on Ψ_c of *C. sake* cells showed similar results. The change in yeast Ψ_c is attributed to the intracellular accumulation/synthesis of polyols, mostly glycerol and arabitol and trehalose.

In molasses media, glycerol and arabitol accumulated at higher levels accounted for efficient osmoregulation. However, the most important finding is the high trehalose accumulation in proline, glucose and sorbitol treatments. Direct uptake of glycerol from the media was also observed. Hallsworth & Magan (1994) pointed out that conidia with elevated trehalose concentrations remained viable for a 17-week storage period for longer that those from control treatments. This study has shown that polyol accumulation may account for better quality, in terms of viability and stress tolerance, of *P. anomala* cells. The high trehalose accumulation levels may have implications for improving shelf-life and perhaps the production of ecologically stable biocontrol agents.

Acknowledgements

This work is part of the European Union project QoL-PL.999-1065 (Biopostharvest) We are grateful to Prof. J. Schnürer for the *P.anomala* J121 strain. Stella Mokiou is grateful to the Foundation Konstantinos Lazaridis for funding.

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Application of RAP-PCR and cDNA-AFLP to isolate genes of *Candida oleophila* (strain O) induced by the presence of galacturonic acid

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Abstract: The yeast *Candida oleophila* (strain O) possesses an antagonistic activity against *Botrytis cinerea* (gray mold) on postharvest apples. This activity has been stimulated by the addition of galacturonic acid (GA) at 0.01 and 0.001 % (w/v) while GA didn't show any protective activity alone. RNA arbitrarily primed polymerase chain reaction (RAP-PCR) and cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP) were used to identify strain O genes induced by the presence of GA. For the RAP-PCR, 82 sets of primers were used, resulting in a total number of 1438 or 1439 bands observed from mRNA of strain O incubated in presence or absence of GA. The average number of bands per primer set was 17.5. Eight bands were selected for their strongest differential expression in presence of GA but were no more differentially observed during repetitions of the RAP-PCR (on the same and/or other batches of mRNA). cDNA-AFLP application with 10 primer sets resulted in the detection of 77 bands in average per primer set and 100 % of reproducibility using the same cDNA batch. One band was differentially expressed in presence of GA. This result remains to be confirmed.

Keywords: *Candida oleophila*, galacturonic acid, RAP-PCR, cDNA-AFLP, biological control, postharvest disease, apple

Introduction

The yeast *Candida oleophila* (strain O) has been isolated from apple (cv. Golden delicious) and selected for its high and reliable antagonistic properties against *Botrytis cinerea* one of the most devastating pathogens of postharvest apples (Jijakli et al., 1999). The addition of galacturonic acid (GA), at 0.01 and 0.001 % (w/v), enhanced the protective activity of strain O (10^5 cfu/ml) to the protective level obtained by strain O applied alone at 10^7 cfu/ml (around 90 %). Furthermore, the GA didn't show any protective activity by itself (Dickburt et al., 2001). In order to isolate strain O genes up-regulated by the presence of this molecule, two molecular techniques were applied to compare mRNA populations extracted from strain O cultivated in YNB + glucose (G) (0.5%) and YNB + G (0.4%) + GA (0.1%). The first technique, the RNA Arbitrarily Primed Polymerase Chain Reaction or RAP-PCR (Sokolov & Prockop, 1994), is based on the retrotranscription and the amplification by PCR of mRNA previously extracted. The amplified fragments are displayed and compared on agarose gel. After mRNA retrotranscription, the second technique, the cDNA Amplified Fragment Length Polymorphism or cDNA-AFLP (Bachem et al., 1996), relies on the digestion of cDNAs by restriction enzymes followed by their ligation with adaptators which serve as priming site during amplification. It allows performing a specific amplification at high stringency, making this technique theoretically more reproducible.

Material and methods

Yeast strain and media

The strain O was cultivated on Potato Dextrose Agar (PDA, Duchefa) and conserved at 4°C during 6 month. Before experiment, it was subcultured on PDA at 25°C. One millilitre of a suspension of strain O (4.10^8 cfu/ml) was inoculated in 50 ml of YNB +G [containing 6.7 g/l of Yeast Nitrogen Base (Gibco) and 5 g/l of G (Merck)]. Six hours later, when the strain O was in exponential phase, 8.10^6 ufc were transferred in 250 ml of differential media (YNB + G or YNB + G+ GA) [containing 6.7 g/l of YNB, 4 g/l of G and 1 g/l of GA (Fluka)]. Flasks were always incubated at 20 °C on a rotary shaker at 120 rpm.

RNA extraction

The yeast cells (10^9 cells per sample) were harvested in exponential phase after 14 hours of incubation in the two differential media by filtration on a 0.45 µm filter (Gelman Laboratories) and resuspended in 1 ml of RNA buffer (0.5 M NaCl; 0.2 M TrisCl pH 7.4; 10 mM EDTA). One millilitre of buffered phenol–chloroform–isoamylic alcohol (PCAi; 25:24:1) and glass beads (425-600 µm, Sigma) were added. After vortexing 10 x 1 min. (with 30 seconds on ice between each vortexing), 3 ml of RNA buffer and 3 ml of PCAi were added. The samples were centrifuged at 5860 g during 10 min. (Rotor GSA, Sorvall RC-5B). Two other extractions with PCAi were carried out on the recuperated aqueous phase. The extract was ethanol precipitated. Samples were DNase treated with 20 U of DNase RNase free (Boehringer Mannheim) in presence of 40 U of RNase inhibitor (Roche).

RAP-PCR

The mRNAs were purified using the batch system of Oligotex mRNA midi kit (Qiagen) and single stranded cDNAs were synthetised following the instructions of the kit "Superscript™ pre-amplification system for the first strand cDNA synthesis" (Invitrogen) using random hexamer primers. The subsequent amplification step was carried out on a UNO II thermocycler (Biometra) as follow: 95°C-15 min., 45 X (94.5°C – 1 min., 34°C – 1min., 72°C – 1min. 30 s), 72°C 10 min. The 50 µl reaction contained 2 µl of cDNA solution (20 ng), 1X PCR buffer and 5 U of Hotgoldstar (Eurogentec), 0.2 mM of each dNTP, 3 mM of $MgCl_2$, 1 mM of each arbitrary decamer primer. The PCR products were analysed by electrophoresis on agarose gel (1,8 %, Difco) in TBE 1X stained by ethidium bromide (100 ppm).

cDNA-AFLP

First strand synthesis of cDNA from total RNA was primed with oligo dT primers and followed directly by second strand replacement synthesis using RNaseH and Taq polymerase I (Superscript™ double stranded cDNA synthesis kit, Invitrogen). The double stranded cDNA was digested with EcoRI and MseI, ligated with EcoRI and MseI adaptators and pre-amplified on a UNO II thermocycler (Biometra) following instructions of the kit "AFLP® analysis system for microorganisms" (Invitrogen). After a 50 fold dilution, the specific amplification was carried out on the same thermocycler with selective Eco and Mse primers, corresponding to the sequence of the adaptators and containing one (Eco+1 and Mse+1) or two (Eco+2 and Mse+2) additional nucleotides. The Eco primer was labelled with [γ^{33}P] ATP. The specific amplification was carried out following instruction of the kit "AFLP® analysis system for microorganisms" (Invitrogen). Two enzymes were tested: the Taq Polymerase (Roche) and the Expand High Fidelity System (Roche). Amplified products were separated by electrophoresis at 100 W during 1.5 h on a denaturing polyacrylamide gel (6 %) containing 7 M urea. Gels were dried on whatman 3MM paper before autoradiography.

Results

RAP-PCR

Eighty-two primer sets were used. The total number of bands observed from mRNA of strain O incubated in YNB + G or YNB + G + GA was 1439 or 1438 respectively. The average number of bands per primer set was 17.5 ranging from 5 to 31 bands per lane. Thirteen bands were only or more intensively expressed in presence of GA. Among these, eight were selected for their strongest differential expression and the corresponding primer combinations were used for a second screening using the same batch of mRNA. Only one band (1000 bp) was still differentially expressed during this second screening. This selected band wasn't more differentially detected in the patterns observed for three different batches of mRNA. By counting the number and the size of the bands for each repetition, the reproducibility was estimated at 65 % when using the same batch of mRNA and 50 % for different batches.

cDNA-AFLP

Three primer combinations, Eco+1/Mse+1, Eco+2/Mse+1 and Eco+1/Mse+2, were assessed. The highest average number of bands per primer combination (77), were obtained with the Eco+1/Mse+2 primer combination. The Expand High Fidelity system was preferred to the Taq polymerase because the observed bands were sharper and of higher intensity. Based on 6 primer sets, revealing a total of 222 bands, a reproducibility of 100 % for the digestion, the ligation and the pre-amplification steps was observed by processing the same batch of ds cDNA at three different times (Figure 1a). Ten primer sets have been tested resulting in 775 and 767 bands for YNB + G and YNB + G + GA respectively. Among those, one band has shown a stronger expression in presence of GA (arrow, Figure 1b).

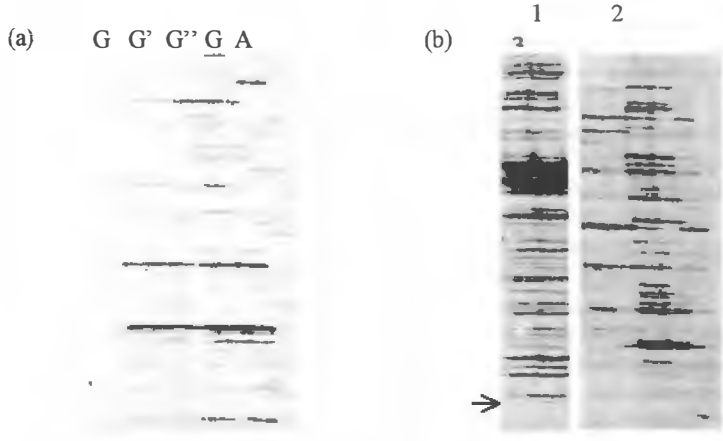

Figure 1. DNA sequencing gel autoradiography of cDNA-AFLP fingerprints from mRNA of strain O cultured in YNB + G (G) and YNB + G + GA (A). (a) Example of results obtained with one primer set by processing the digestion, the ligation and the pre-amplification steps at three different times from the same cDNA sample. (b) Example of fingerprint obtained with 3 primer sets (1, 2, 3). Positive control (C): DNA from *E. coli*. → : band specifically observed in presence of GA in the culture media.

Discussion

The RAP-PCR is quick and easy to perform. Nevertheless, its lack of reproducibility (only 50 % for different batches of mRNA) represents its major drawback. Depending of the model, the reproducibility of differential display has already been evaluated at 60 % (Bauer et al., 1994) and 45 % (Kuhn, 2001). The RAP-PCR was used to compare mRNA populations expressed by the strain O in YNB + G or YNB + G + GA. Among 8 differentially expressed bands from our first screening, no one has shown a reproducible differential expression after further repetitions. The cDNA-AFLP was also applied on the same model after some optimization (primer combinations and polymerase selection). Our preliminary results indicate a better reproducibility for the cDNA-AFLP as already described by Jones & Harrower (1998) and Gellatly *et al.* (2001). After testing 10 primer pairs, one band of interest has been observed. The screening will be completed and the experiment will be repeated for the bands of interest. Bands reproducibly observed will be characterized. Furthermore, thanks to the weak amount of total RNA needed, the cDNA-AFLP could be applied *in vivo* on apple.

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Evaluation of potential agents for postharvest biocontrol of *Alternaria alternata* in tomato

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Abstract: The biocontrol activity of ten Colombian native yeast isolates was evaluated against *Alternaria alternata* in harvested tomatoes kept stored at 6±1°C and at 17±2°C. In one assay, wounds were inoculated with yeast isolates (10^7 cells/ml), 24 hours before inoculation with *A. alternata* (10^6 conidia /ml); all yeast isolates significantly reduced diameter of lesion at 17°C, and eight strains did so at 6°C. *Pichia onychis* (Lv027) exhibited the highest biocontrol activity. However, when the protection test with strain (Lv027) was repeated, considerable variation was found in disease reduction and in pathogen aggressiveness. Additionally, disease was significantly reduced when different concentrations of the pathogen (10^6, 10^5 and 10^4 conidia/ml) were evaluated in interaction with *P. onychis* applied at 10^7 cells/ml. In a second experiment, application of the 10^7 cells/ml of yeast at 48 h before the pathogen at 10^6 conidia/ml gave significantly higher protection than when it was applied 24 h before the pathogen.

Key words: *Alternaria alternata, Pichia onychis,* tomato, postharvest, biological control

Introduction

Alternaria alternata can cause both quiescent infection before harvest and aggressive disease after harvest. Disease development is related to the presence of wounds, and injuries from sunscald, excessive heat and frost injuries (Rotem, 1994). At experimental or commercial level, several pre- and postharvest measures have been proposed to reduce the incidence and severity of *A. alternata*. The main method is preharvest fungicide applications, but the level of control is inadequate due to development of resistant pathotypes of *A. alternata* (Biggs, 1995). Further, there are concerns regarding public health and environmental hazards associated with high levels of pesticides in orchards. More acceptable control methods such as possible biocontrol is needed. In previous studies, in Colombia, the yeast *Pichia onychis* (Lv027) isolated from onion was found to be effective against *Botrytis allii* in onion and *Rhizopus stolonifer* on tomato (García et al., 2001). In the present study, the biocontrol activity of *P. onychis* and nine other yeasts native to Colombia were evaluated against *A. alternata* on tomato.

Materials and methods

To evaluate biocontrol activity of ten yeast isolates obtained from the Biocontrol Laboratory Collection, two wounds (6 mm wide and 3 mm deep) were made at the equator point of surface disinfested tomato fruits. Each wound was treated immediately with 25 µl of yeast suspension (10^7 cell/ml). After 24 h, wounds were each inoculated with *A. alternata* (25 µl of a 10^6 conidia/ml suspension). There were 15 fruits per treatment in a complete randomized

design in each test. The fruits were incubated at 6°C and 17°C. Diameter of lesion were measured at 20 and 24 days after inoculation in tomatoes incubated at 17°C and 6°C respectively. Results were expressed as disease severity activity. With the yeast selected for its highest biocontrol activity, the assay was repeated twice, by using the same methodology described above. In order to determine the effect of pathogen concentration on the biocontrol effectiveness of the selected yeast (*P. onychis* Lv027), fruit wounds were inoculated with 25 µl of suspension (10^7 cells/ml) of the yeast and 24 hours later were inoculated with 25 µl of three pathogen suspensions containing: 10^4 conidia/ml, 10^5 conidia/ml and 10^6 conidia/ml of *A. alternata*.

To determine the effect of time interval between yeast and pathogen inoculation on biocontrol activity of *P. onychis*, tomato wounds were inoculated with 25 µl of a suspension containing 10^7 cells/ml and incubated 24 h or 48 h before *A. alternata* (25 µl of a 10^6 conidia/ml suspension) was inoculated. Experimental conditions, and the results evaluation were as described above.

Results and discussion

When the biocontrol effectiveness of ten native yeasts was evaluated on tomato incubated at 6°C and 17°C, significant disease control was achieved with *P. onychis* (Lv027) at both temperatures, 24 and 20 days after the pathogen inoculation on tomatoes incubated at 6°C and 17°C, in the inoculated control the lesion diameter (disease severity) was 33.2 mm and 58.8 mm respectively, while in *P. onychis* (Lv027) treatment it was of 8.1 mm and 5.7 mm respectively (Table 1).

Table 1. Biocontrol activities of ten yeast isolates against *A. alternata* (10^6 conidia/ml) in tomatoes, at 6±1°C after 24 days and at 17±2°C after 20 days

Treatment	Lesion diameter (mm) at 6°C	Lesion diameter (mm) at 17°C
Control (pathogen without yeast)	33.2 a	58.8 a
Lv090	24.5 b	45.9 ab
Lv031	26.7 b	44.9 ab
Lv050	27.4 b	42.9 bc
Lv146	26.9 b	36.6 bc
Lv389	26.1 b	35.8 bc
Lv198	28.0 b	34.9 bc
Lv064	26.8 b	34.8 bc
Lv077	26.7 b	29.8 c
Lv240	25.3 b	28.9 c
Lv027	8.1 c	5.7 d

Columns with the same letter do not present meaningful differences according to Tukey's test.

The high biocontrol activity of *P. onychis* agrees with previous studies on onions and tomatoes, which show high protection levels more than 85% at 6°C and 17°C (García et al., 2001). This result is promising taking into account that previous studies showed difficulties in obtaining efficient biocontrol of *Alternaria* sp.; for example, *Candida sake* reduced populations of *Cladosporium* sp. and *Penicillium* sp on apple surface, however, no significant effects were observed in *Alternaria* sp. (Teixidó et al., 1999).

When biocontrol assay was repeated twice using the yeast *P. onychis* (Lv027), its biocontrol activity decreased at both temperatures (Table 2), which was expressed as a significant increase in the lesion diameter produced by the pathogen. However in the absence

of the yeast at both temperatures, the lesion diameter produced by *A. alternata* significantly increased as compared to the first repetition. This phenomenon could be mainly caused by an increase in the pathogen aggressiveness, which could be due to recurrent pathogen inoculations on tomato fruit tissue. It is possible to suggest that due to heterokaryotic nature of *A. alternata* (Biggs, 1994), its constant inoculations in vegetal tissue have selected more virulent nuclei.

Table 2. Biocontrol activity in the time of the yeast *P. onychis* (Lv027) against *A. alternata* at 6°C and 17°C

	6°C Lesion diameter (mm)		17°C Lesion diameter (mm)	
	Control	*P. onychis* + *A. alternata*	Control	*P. onychis* + *A. alternata*
Time 1	29.6 b	7.2 c	43.9 c	2.3 b
Time 2	37.4 a	34.2 a	59.4 a	39.5 a
Time 3	36.3 a	31.7 b	55.3 b	40.9 a

Columns with the same letter do not present meaningful differences according to Tukey´s test

In the presence of *P. onychis* (Lv027), disease severity was significantly reduced in tomatoes inoculated with 10^6, 10^5 and 10^4 conidia/ml of *A. alternata*; the highest reduction was achieved when pathogen was inoculated with 10^5 and 10^4 conidia/ml with 27.7 mm and 25.1 mm lesion diameter respectively in comparison with the non treated control with 55.1 mm and 43.1 mm lesion diameter respectively (Figure 1).

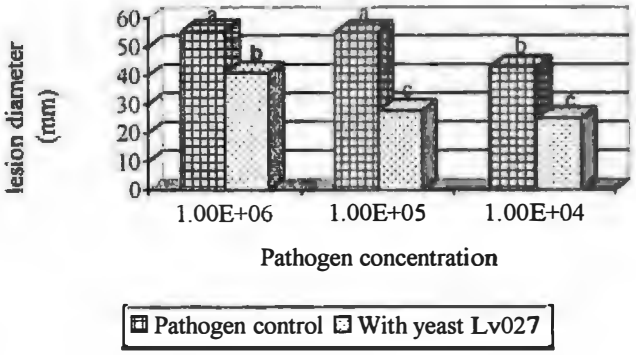

Figure 1. Effect of *A. alternata* concentration on the *P. onychis* (Lv027) biocontrol activity, after 16 days at 17°C. Values with the same letter are not significantly different according to Tukey's test.

When two time intervals application between yeast *P. onychis* (Lv027) and *A. alternata* were evaluated, with both time intervals (24 h and 48 h) significantly reduced lesion diameter (40.9 mm and 17.4 mm) were observed compared with the control (55.3 mm) after 16 days (Figure 2). However, the highest reduction in lesion diameter was obtained when *P. onychis* was inoculated 48 h before *A. alternata*. Previous inoculation of the biocontrol agent could

allow yeast growth at the wound sites, which could have limited *A. alternata* development. Importance of previous inoculation of antagonists in biocontrol of postharvest diseases produced by other pathogens, was also established by Quing & Shiping (2000), who showed that *Pichia menbranefaciens* inoculated 48 h before *R. stolonifer* could utilize available nutrients and colonize wound surface.

Based on these results, we suggest that the selected yeast could be promising for biocontrol of *A. alternata*.

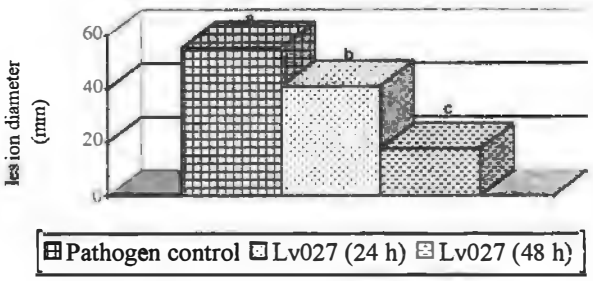

Figure 2. Biocontrol effect of two time intervals (24 h and 48 h) between *P. onychis* Lv027 (10^7 cells/ml) against *A. alternata* inoculations, after 16 days at 17°C. Values with the same letter are not significantly different according to Tukey's test

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Effects of abiotic and biotic factors on *Trichoderma* strains with biocontrol potential

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Abstract: The effects of low temperature, low water potential, pesticides, heavy metals and antagonistic bacteria on *Trichoderma* strains with biocontrol potential were examined *in vitro*. Cold-tolerant strains growing well at 5°C were isolated from soil samples. In dual culture tests at 10°C, most strains antagonized plant pathogenic fungi. The activities of extracellular enzymes important for mycoparasitism were significant at 5°C in the cold-tolerant strains. Nearly linear correlation was found between water potential and colony growth rate with higher growth rates at higher water potential. Secretion of the enzymes also depended on the water potential of the liquid media. However, significant *in vitro* activities were measured for most of the enzymes even at water potential values below the limit of mycelial growth. The effects of seven pesticides on *Trichoderma* strains were also examined. The fungicide susceptibility of the strains may cause problems during their combined application with antifungal compounds. For such purposes fungicide resistant mutants should be applied. The examined heavy metals showed inhibition of mycelial growth, but the extracellular enzyme systems of *Trichoderma* strains remained active even at heavy metal concentrations, where mycelial growth was already strongly inhibited. Heavy metal resistant mutants were isolated by UV-mutagenesis and tested for possible cross-resistances. Some of the mutants were effective antagonists of plant pathogenic fungi even on media containing the respective heavy metals. Eighteen *Trichoderma* isolates were tested for their ability to degrade heat-inactivated Gram-positive and Gram-negative bacteria. In addition to trypsin-like protease, chymotrypsin-like protease and β-1,4-*N*-acetyl-glucosaminidase enzymes, muramidase-like activities were also present in the induced ferment broth of a *T. harzianum* strain.

Key words: *Trichoderma*, biocontrol, environmental factors, extracellular enzymes

Introduction

Trichoderma species are imperfect fungi, with teleomorphs belonging to the ascomycete order *Hypocreales*. Their mycoparasitic ability against plant pathogenic fungi allows for the development of biocontrol strategies based on *Trichoderma* strains (Manczinger, 1999; Manczinger et al., 2002). Such strategies can be incorporated in a complex integrated plant protection. When planning the application of biocontrol strains, it is very important to consider the environmental stresses affecting microbial activities. Low temperature, low water potential, the presence of heavy metals or pesticides and antagonistic bacteria in the soil are among the most important stress factors. The study of the influence of such parameters on *Trichoderma* strains is of great importance: biocontrol strains should have better stress tolerance levels than the plant pathogens against which they are going to be used for biocontrol.

Materials and methods

Strains

The 360 *Trichoderma* strains were isolated from Hungarian soil samples. *Fusarium culmorum*, *Fusarium oxysporum* f. sp. *dianthi*, *Pythium debaryanum* and *Rhizoctonia solani* strains were obtained from the strain collection of the Cereal Research Non-Profit Company, Szeged. The strains of bacteria (*Bacillus cereus* var. *mycoides*, *Bacillus megaterium*, *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Serratia marcescens*) used in the experiments were derived from the Microbiological Collection of the University of Szeged (SZMC).

Experimental procedures

Culture media and methods applied for the investigation of the effects of low temperature (Antal et al., 2000), water potential (Kredics et al., 2000), heavy metals (Kredics et al., 2001a, b) and antagonistic bacteria (Manczinger et al., 2002) (colony growth measurement, test of *in vitro* antagonism, measurement of enzyme activities, isolation and characterization of mutants, testing of bacterium-degrading ability, Sephadex G-150 column chromatography, etc.) were described earlier. Pesticides (benomyl, MBC, dicloran, fenuron, fluometuron, monuron and diuron) derived from Aldrich. Susceptibility testing was performed on solid medium (1% glucose, 0.5% KH_2PO_4, 0.1% $NaNO_3$, 0.2% yeast extract, 0.1% $MgSO_4 \times 7H_2O$) supplemented with pesticides in different concentrations in the following ranges: benomyl 0-2 μg/ml, MBC: 0-2 μg/ml, dicloran: 0-10 μg/ml, fenuron: 0-700 μg/ml, fluometuron: 0-1000 μg/ml, monuron: 0-1000 μg/ml, diuron: 0-50 μg/ml.

Results and discussion

Cold tolerance of biocontrol* Trichoderma *strains

Of 360 *Trichoderma* strains investigated, 14 - identified as *T. aureoviride*, *T. harzianum* and *T. viride* - grew well at 5°C on both minimal and yeast extract agar media. The incidence of cold-tolerant isolates was the highest in species group *T. viride*. In dual culture tests at 10°C, all cold tolerant strains produced appressoria and antagonized the plant pathogens *R. solani* and *F. oxysporum* f. sp. *dianthi*. *T. aureoviride* and *T. viride* strains were more effective against *P. debaryanum* than *T. harzianum* strains. The activities of extracellular β-1,4-*N*-acetyl-glucosaminidase (NAGase), β-glucosidase and trypsin- and chymotrypsin-like proteases - thought to be involved in the mycoparasitic process - were also examined and results showed that these enzymes were highly active at 5°C in the cold-tolerant strains (Antal et al., 2000).

Influence of water potential on strain* Trichoderma harzianum *T66

Influence of water potential on linear mycelial growth, secretion and *in vitro* activities of β-glucosidase, cellobiohydrolase, β-xylosidase, NAGase and chymotrypsin-like protease enzymes of the cold-tolerant *T. harzianum* strain T66 was studied at different temperatures (Kredics et al., 2000). Nearly linear correlation was found between water potential and colony growth rate at both 25°C and 10°C with higher growth rates at higher temperature and water potential. Optimal water potential values for the secretion of β-glucosidase, cellobiohydrolase, β-xylosidase, NAGase and chymotrypsin-like protease enzymes were different. Cellobiohydrolase and NAGase enzymes showed optimal secretion at the highest examined water potential, while the maximum activities of secreted β-glucosidase, β-xylosidase and chymotrypsin-like protease enzymes occurred at lower water potential values than those

optimal for growth. The *in vitro* enzyme activities were affected by water potential, but significant enzyme activities were measured for most of the enzymes even at -14.8 MPa that is below the water potential, where mycelial growth ceased. These results suggest the possibility of using mutants with improved xerotolerance for biocontrol purposes in soils with lower water potential.

Effects of pesticides and heavy metals on biocontrol* Trichoderma *strains

The effects of three fungicides (benomyl, MBC, dicloran) and four herbicides (fenuron, fluometuron, monuron, diuron) were examined on the growth of *T. aureoviride* T122, *T. harzianum* T66 and T334, and *T. viride* T124 and T228 strains. The IC_{50} concentrations are presented in Table 1. In the case of diuron, 50% inhibition could not be reached, and the IC_{50} concentrations were found to be so high for the other herbicides, which values can not be present in the soil during their application. However, the fungicide susceptibility of the strains may cause problems during their combined application with antifungal compounds. For such purposes fungicide resistant mutants should be applied.

Table 1. IC_{50} concentrations of the examined pesticides in μg/ml. ND: not determinable

Strain	benomyl	MBC	dicloran	fenuron	fluometuron	monuron
T66	0.52	0.26	9.75	385.00	200.00	170.00
T114	0.38	0.21	11.10	618.00	580.00	430.00
T122	0.45	0.22	ND	580.00	430.00	275.00
T124	0.48	0.21	12.25	515.00	575.00	340.00
T228	0.45	0.24	7.80	515.00	535.00	215.00
T334	0.41	0.21	ND	420.00	380.00	325.00

The effect of ten metals (aluminium, copper, nickel, cobalt, cadmium, zinc, manganese, lead, mercury and iron) on mycelial growth and on the *in vitro* activities of trypsin-like protease, chymotrypsin-like protease, NAGase, β-1,3-glucanase, β-glucosidase, cellobiohydrolase, β-xylosidase and endoxylanase enzymes was also investigated in the case of these six strains (Kredics et al., 2001a, b). Mycelial growth was significantly influenced by the heavy metals. The lowest IC_{50} values were found for copper, while the highest were for aluminium. In a concentration of 1 mmol only mercury inhibited the examined extracellular enzymes significantly, in the case of the other metals the enzymes of *Trichoderma* could remain active even at concentrations inhibiting mycelial growth, suggesting that breeding for heavy metal resistant *Trichoderma* strains could result in biocontrol agents effective against plant pathogenic fungi even under heavy metal stress.

A total number of 177 metal resistant mutants were isolated by UV-mutagenesis and tested for possible cross-resistances (Kredics et al., 2001b). Significant cross-resistance was found in the case of the aluminium- and nickel-resistant mutants to copper and in the case of copper resistant ones to nickel. Some of the mutants were effective antagonists of plant pathogenic *F. culmorum*, *F. oxysporum* f. sp. *dianthi*, *P. debaryanum* and *R. solani* strains even on media containing the respective heavy metals. Such mutants might be the preferred choice for combined application with heavy metal-containing pesticides in the frame of a complex integrated plant protection.

Bacterium-degrading ability of biocontrol* Trichoderma *strains

Eighteen *Trichoderma* strains were screened for their ability to degrade bacterial cells (Manczinger et al., 2002). The specificity spectrum and the intensity of degradation were highly variable. In the case of five strains showing outstanding degrading abilities towards *B.*

subtilis, the NAGase, trypsin-like and chymotrypsin-like protease activities were determined under inductive and non-inductive circumstances. All strains were able to produce NAGase and proteases constitutively at a moderate level, which could be elevated by induction with *B. subtilis* cells: 3-6 times more NAGase and proteases were produced in inductive media.

The inductive ferment broth of an outstanding strain, *T. harzianum* T19, was fractionated on a Sephadex G-150 column. The strain produced at least 3 trypsin-like proteases, 6 chymotrypsin-like proteases, and 4 NAGases upon induction with *B. subtilis* cells. Muramidase-like activities were also present in the ferment broth of this *T. harzianum* strain. These results indicate that bacterium-degrading ability is common, but highly variable among *Trichoderma* strains. Proteases, NAGases and muramidases seem to have great importance in the degradation of bacterial cells.

In addition to testing their ability to antagonize plant pathogenic fungi, the determination of their bacterium-degrading capabilities may also be useful in the evaluation of biofungicide *Trichoderma* strains, as perhaps this property can help the strains to be dominant microorganisms in the habitats where they are applied.

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Biocontrol of *Rhizoctonia solani* (Kühn.) in Anatolia black pine *Pinus nigra* subsp. *pallasiana* (Lamb Holmboe) seedlings in Turkey

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Abstract: The efficacy of two soil-borne *Bacillus subtilis* strains and Planter Box (*Trichoderma harzianum* Rifai T-22), a biological fungicide, was investigated under *in vitro* and *in vivo* conditions against five different pathogenic *Rhizoctonia solani* isolates obtained from diseased Anatolia Black Pine seedlings. The colonial growth of five different *R. solani* isolates was inhibited at the ratios of between 75-90 % and 95-100 % by using *B. subtilis* isolates and Planter Box, respectively. Rizolex (Tolchlofos - methyl) was more effective (100 %) than those of the both treatments. In *in vivo* studies, the inoculations of *B. subtilis* strains and Planter Box were achieved by seed coating after soil infestation with *R. solani*. The success of *B. subtilis* isolates and Planter Box was compared with that of Rizolex, a commonly used fungicide for the control of *Rhizoctonia* disease in Turkey. The treatments of the *B. subtilis* isolates and Planter Box reduced disease caused by five different *R. solani* isolates at the ratios of between 75.76 % and 56.90 % in *in vivo* experiments. It was determined that the biological treatments were as effective as fungicide treatment to control disease caused by different *R. solani* isolates The effects of Rizolex were at the ratios of between 67.97% and 48.28 %. *B. subtilis* and Planter Box seem to be promising candidates for biocontrol of root rot disease caused by *R. solani* on Anatolia Black Pine seedlings in nursery.

Key words: Damping-off, *Rhizoctonia solani*, Planter Box, *Bacillus subtilis*, *Pinus nigra* subsp. *pallasiana*

Introduction

It is prudent to ensure a continued wood supply through successful regeneration, maintenance and management of the forests in order to maximise economic benefits from forestry. Approximately 700 million forest seedlings are produced each year in Turkey (Anonymous, 1995). Successful tree seedling production depends on many interacting factors in nurseries. Seedling quality is reflected on the subsequent tree growth and the forest productivity. Consequently, the production system should be aimed at producing healthy and well developed seedlings. However, the intense production systems operating in forest nursery environments make the seedlings particularly sensitive to fungal pathogens which can cause great losses both before and after planting out. Serious damage is primarily caused by a variety of damping-off pathogens, including *Alternaria alternata*, *Botrytis cinerea*, *Cladosporium* spp., *Fusarium* spp., *Macrophomina phaseolina*, *Phytophthora* spp., *Pythium* spp., *Rhizoctonia solani* (Sandlin & Ferrin, 1993; Viljoen et al., 1994; Chin, 1995; James et al., 1995; Motta et al., 1996; Belisario et al., 1997; Lilja et al., 1997; Ozdamar & Turhan, 1999). These pathogens are responsible for considerable economic losses (Bloomberg, 1973; Sutherland et al., 1989; Hiratsuka et al., 1995; Paige et al., 1995).

Material and methods

In this work, five pathogenic *R. solani* isolates (SÇK-1, SÇK-2: Seydisehir Black pine isolates; EÇK-1, EÇK-2: Egirdir Black Pine isolates; GÇK-1: Golhisar isolates) obtained from diseased Anatolia Black Pine seedlings, two soil-borne *B. subtilis* isolates (BB-2, AB-27), a commercial bio-fungicide, Planter Box, and Tolchlophos-methyl (Rizolex) were used. *P. nigra* subsp. *pallasiana* seeds originated from Egirdir were used *in vivo* studies. In *in vitro* studies, BB-2 and AB-27 isolates of *B. subtilis*, Planter Box and Rizolex were added into petri dishes containing PDA (Potato-Dextrose-Agar) medium at proper temperature and dosages (Basım et al., 1999). After two days, the discs of R. solani in the size of 4 mm were placed in the middle of the petri dishes and evaluation was done when the pathogen fungus has completely covered petri dishes containing only PDA medium (Control). The effects of the treatments were determined by comparing dose of the control petri dishes. *In vivo* experiments were conducted according to Özdamar & Turhan (1999). Fifteen replicates were used for each treatment. Disease severity was determined after 30 days from sowing. Tarist statistics programme was used and Duncan test was made for all treatments.

Results and discussion

Antagonistic activity of Planter Box and inhibiting activity of Rizolex against five *R. solani* isolates were found to be effective treatments in *in vitro* studies (Table 1). According to the results obtained through measuring the colonial ratio of *R. solani*, all isolates were affected by the Rizolex and Planter Box. While Rizolex completely suppressed the colonial growth of pathogenic isolates, Planter Box reduced the colonial growth of *R. solani* isolates at the ratio of 98.22%. Considerable reduction in the colonial area of the *R. solani* isolates by BB-2 and AB-27 was found to be 90.21 % and 95.11 %, respectively (Table 1).

Table 1. The inhibitory effect of treatments on the mycelial growth of the *R. solani* isolates. The average was calculated from five replicates.

	BB-2	**AB-27**	**Rizolex**	**Planter Box**
SÇK-1	90.218	90.884	100	93.774
SÇK-2	86.886	89.996	100	98.222
EÇK-1	75.994	76.66	100	97.554
EÇK-2	79.774	79.996	100	90.442
GÇK-1	89.552	95.11	100	97.554

The protective value of the treatments in pot soils was found to be more effective in Planter Box and Rizolex treatments than those of the others. Disease severity was between 100% and 93.33% in controls. The disease occurred in pot treatment was decreased at the ratios of 23.02% , 29.89%, 36.56 % and 49.24 % by Planter Box, Rizolex, AB-27, BB-2, respectively (Table 2). Planter Box treatment significantly reduced the disease comparing to other treatments, except EÇK-2 isolate in *in vivo* experiments (Table 2). The results obtained from *in vitro* and *in vivo* experiments seemed to be correlated with each other in this study. These results suggest that Planter Box is effective in reducing *R. solani* in Anatolia black pine seedlings in nursery and can be a practical method of managing fungal pathogens in forest nurseries. Soil sterilization with methyl bromide and other fumigants is traditionally used to control fungal pathogens in forest nurseries. These control methods are effective but have a number of disadvantages. They are expensive, potentially toxic to animals and humans,

contribute to depletion of stratospheric ozone and inhibit communities of beneficial microbes, including mycorrhizal fungi. Additionally, these fungicides have become less effective due to the development of pathogen resistance (Ogawa et al., 1981; Sidhu & Chakravarty, 1990). Moreover, if the disinfection is not well made diseases are only postponed. An integrated, biological-based disease control approach is necessary in order to protect the environment and human health through a reduction in the use of methyl bromide and other fumigants (Schwerdtfeger, 1981). A biocontrol agent such as *T. harzianum* could be beneficial within an integrated management system in forest nurseries. *B. subtilis* and Planter Box seemed to be promising candidates for biocontrol of damping-off disease caused by *R.solani* on Anatolia Black Pine seedlings in nurseries as mentioned previously (Bası m et. al., 1999; Bası m et al., 2001).

Table 2. Disease severity and disease reduction on Anatolia Black pine treated with biological agents and Rizolex on infested soil by *R. solani* isolates *in vivo*

Treatmen	Disease severity (%)					Disease reduction (%)				
	SÇK-1	SÇK-2	EÇK-1	EÇK-2	GÇK-1	SÇK-1	SÇK-2	EÇK-1	EÇK-2	GÇK-1
Control	94.99 a*	93.33 a	100.00 a	100.00 a	96.66 a	-	-	-	-	-
BB-2	53.27 b	49.64 b	68.33 b	68.33 b	66. 66 b	43.91	46.81	46.34	32.14	31.03
AB-27	36.56bc	36.51 b	53.43bc	53.43 b	60.04 bc	61.51	60.88	46.57	37.50	38.19
Rizolex	33.23bc	34.84 b	43.27 c	41.56 c	44.99 c	65.01	62.67	56.73	67.97	48.28
P. Box	23.02 c	33.13 b	41.56 c	43.27 c	41.66 c	75.76	64.50	58.44	60.82	56.90

* Means within a column with a letter in common are not significantly different according to Tarist statistic programme ($P \leq 0,05$).

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The *in vitro* biological action of pesticides on the biocontrol agent *Trichoderma viride*

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Abstract: The *in vitro* action of 45 pesticides (5 fungicidal mixtures, 6 insecticidal mixtures, 11 insecto-fungicidal mixtures and 23 herbicides) manufactured by Oltchim S.A.-Rm. Valcea (Romania) has been tested against the antagonistic fungus *Trichoderma viride* (isolate Td 50). All 5 tested fungicides (Tiracarb 60 PTS, Tiracarb 600 SC, Tiramet 60 PTS, Tiramet 600 SC, FRV 12 SC) acted as a strongly inhibitory (toxic) to *T. viride*. Among 11 insecto-fungicides, 4 products (Tirametox 90 PTS, Tirametox 625 SC, Supercarb T 80 PSU, Supercarb T 585 SC, Procarb L, Trialin MT) proved a high toxic activity against *T. viride*, 3 insecto-fungicides showed a strong inhibitory to moderate inhibitory action and only two insecto-fungicides (Tebuconazole + Lindane, FRV 1A PTS) were moderately inhibitory to *T. viride*. All 6 tested insecticides proved moderately inhibitory activity to *T. viride*, 3 of them (Oleoekalux + Quinalfos-1997, US 1-RV, Lindan 400SC) acting as moderately inhibitory to slight inhibitory. Among the 23 herbicides, 4 products (Butizin 600 SC, Mecloran 48 CE, Oltisan Extra, Olticarb 75 CE) showed a strong inhibitory activity and one (Sanolt Combi 400 SE) was toxic to moderately toxic. Other 5 herbicides (Butiran 1:1, Diburom 800 CE, Diizocab 80 CE, Mecloran 35 CE, Oltest CE) acted as moderately inhibitory and 8 products (Butizin 40 SC, Oltisan M, 2,4 SDMA 600 RV, Zinal 500 SC, FRV 04 CE, FRV 05 LC, FRV 09 CE, Select Super RV) showed a moderately inhibitory to slightly toxic activity. A very important group of 4 herbicides (Banvel 600 LC, FRV 06 LC, Romaxin 500 SC, Romanex 500 SC) was slightly toxic and only one (Icedin Super RV) was selective to *T. viride*. Based on our experimental results, a clasiffication of pesticides' biological action on *T. viride* in 5 categories has been built. All the pesticides revealing a moderately inhibitory to slightly toxic action and the selective ones (3 insecticides, 13 herbicides) showed increased practical importance, being efficient in crop protection and not inducing imbalances to the agroecosystem where applied.

Key words: *Trichoderma viride* biocontrol agent, biological action of pesticides

Introduction

In the plant protection literature a series of data is known, concerning the action of chemicals on the antagonistic fungi from the genus *Trichoderma* (Baicu, 1982, 1987, 1990; Elad, 1991; Şesan, 1985, 1986; Şesan et al., 1998; Şesan & Oprea, 1998, 1999, 2001). The present report characterizes the biological activity of 45 plant protection products, developed by the main Romanian chemical plant – Oltchim S.A.–Rm. Valcea (Romania) – on the antagonistic fungus *Trichoderma viride* Pers. Ex S. F. Gray, one of the most important biocontrol agent against plant diseases.

Material and methods

The *in vitro* action of 45 pesticides, 5 fungicidal mixtures, 6 insecticidal mixtures, 11 insecto-fungicidal mixtures and 23 herbicides manufactured by Oltchim S.A.-Rm. Valcea – Romania has been tested against the antagonistic fungus *Trichoderma viride* (isolate Td 50), used as

one of the most spread and efficient biocontrol agent. The method used was inclusion of the products in a nutritive medium (PDA – potato-dextrose-agar) at the rate recommended by the *Codex of plant protection products registered for use in Romania* (1999) in three successive halved dilutions. Observations consisted in measuring the diameter of the test fungi colonies until they completely covered the surface of the culture medium of Petri dishes in the check variant (without pesticides).

Rating has been performed by calculation of inhibition (I %), lethal concentrations (LC 50, LC 90), regression line equations (RLE) and correlation coefficients (Ccf) by the method of dose-logarithm, mortality-probit. The used abbreviations for the biological activity of pesticides were: strongly inhibitory or toxic (T), strongly inhibitory with tendency to moderate toxicity (T-MT), moderately inhibitory (MT) and slightly inhibitory (ST) or non-toxic, even selective (Sel.).

Results and discussion

All the 5 tested fungicides proved to be toxic to *Trichoderma viride* (Table 1) as it is shown by the high values of the inhibition percent and by the calculated correlation coefficients. The insecticides Oleoekalux (Olkx) + Quinalfos (Qfos) 3%-1998, Lindan 666 SC, Applaud + Mineral Oil had a moderately toxic action on *T. viride*, while the other three insecticides (Oleoekalux + Quinalfos 3%-1997, Lindan 400 SC, US 1–RV) had a moderate to slight toxicity (Table 2). Among the 11 tested insecto-fungicides, 6 products (Tirametox 90 PTS, Tirametox 625 SC, Supercarb T 80 PSU, Supercarb T 585 SC, Procarb L, Trialin MT) were toxic to *T. viride*, 3 products [Diniconazol (Din.) + Lindan, Gammavit 85 PSU, Trialin] had a toxic to moderate-toxic behaviour, and 2 products [Tebuconazole (Teb.) + Lindan, FRV 1A PTS] proved a moderate toxicity (Table 3). The 23 performed herbicides proved the following behaviour: 4 of them were toxic (Butizin 600 SC, Mecloran 48 CE, Oltisan Extra, Olticarb 75 CE), one (Sanolt Combi 400 SE) was toxic to moderately toxic, 5 products (Butiran 1:1, Diburom 800 CE, Diizocab 80 CE, Mecloran 35 CE, Oltest 500 CE) were moderately-toxic, 8 products (Butizin 40 SC, Oltisan M, 2.4 SDMA 600 RV, Zinal 500 SC, FRV 04 CE, FRV 05 LC, FRV 09 CE, Select Super RV) proved a moderate to slight toxicity, 4 products (Banvel 600 LC, FRV 06 LC, Romazin 500 SC, Romanex 500 SC) behaved as slightly toxic and only one (Icedin Super RV) was selective for *T. viride* (Table 4).

Table 1. *In vitro* biological action of fungicides on *Trichoderma viride*

Fungicides	Concs.	I (%)	RLE	LC 0mg/l	LC 90mg/l	Ccf	Activity
Tiracarb 60 PTS	0.2-0.025	99.3-82.8	y = 9.64 + 1.92x	0.00390	0.0217	0.817	T
Tiracarb 600 SC	0.2-0.025	99.3-99.3	y = 9.64 + 1.92x	0.00390	0.0217	0.817	T
Tiramet 60 PTS	0.2-0.025	99.3-85.7	y = 9.39 + 1.72x	0.00282	0.0156	0.812	T
Tiramet 600 SC	0.2-0.025	99.3-84.3	y = 9.52 + 1.82x	0.00336	1.6878	0.814	T
FRV 12 SC	0.2-0.025	91.7-89.0	y = 6.15 + 0.17x	4874458	0.1955	0.991	T

Table 2. *In vitro* biological action of insecticides on *Trichoderma viride*

Insecticides	Concs	I (%)	RLE	CL 50mg/l	CL90mg/l	Ccf	Activity
Olkx+Qfos('97)	0.2-0.025	70.0-17.1	y = 6.78 + 1.58x	0.02744	0.4807	0.954	*MT-ST*
Olkx+Qfos('98)	0.2-0.025	72.4-58.4	y = 5.69 + 0.15x	0.00003	4679.5	0.962	*MT*
US 1 - RV	0.2-0.025	68.6-37.1	y = 6.10 + 0.92x	0.06431	1.5382	0.987	*MT-ST*
Lindan 400 SC	0.2-0.025	67.3-22.8	y = 6.27 + 1.14x	0.07671	1.0117	0.927	MT-ST
Lindan 666 SC	0.2-0.025	85.7-65.7	y = 6.65 + 0.73x	0.00572	0.3144	0.960	MT
Appl. + Min. oil	0.2-0.025	73.4-16.7	y = 7.49 + 2.37x	0.08903	0.3083	0.901	*MT*

Table 3. Biological action of insecto-fungicides on *Trichoderma viride*

Insecto-fungicides	Concs.	I (%)	RLE	CL50mg/l	CL 90 mg/l	Ccf	Activity
Din.+Lindan	0.2-0.025	99.3-83.6	y = 8.54 + 1.68x	0.0077	0.0044	0.830	T-MT
Teb.+Lindan	0.2-0.025	83.6-53.8	y = 6.72 + 0.95x	0.0156	0.3394	0.951	MT
Tirametox 90PTS	0.3-0.035	99.8-99.6	y = 7.95 + 0.22x	<0.010	<0.100	0.973	T
Tirametox 625SC	0.3-0.050	91.7-88.9	y = 6.15 + 0.17x	487445	0.1955	0.991	T
Gammavit 85PSU	0.2-0.025	99.7-57.1	y = 7.31 + 1.36x	0.0204	0.1764	0.709	T-MT
SupercarbT 80PSU	0.2-0.025	99.7-99.5	y = 7.89 + 0.21x	<0.100	<0.010	0.931	T
Supercarb T 585SC	0.2-0.025	91.7-89.0	y = 6.15 + 0.17x	467445	0.1955	0.991	T
Procarb L	0.2-0.025	99.7-99.4	y = 7.99 + 0.28x	<0.100	<0.010	0.973	T
Trialin	0.2-0.025	97.0-75.5	y = 7.77 + 1.31x	0.0077	0.0730	0.978	T-MT
Trialin MT	0.2-0.025	99.7-99.4	y = 7.93 + 0.24x	<0.100	<0.010	0.997	T
FRV 1A PTS	0.2-0.025	88.1-58.8	y = 7.29 + 1.45x	0.0265	0.2008	0.959	MT

Table 4. Biological action of herbicides on *Trichoderma viride*

Herbicides	Concs	I (%)	RLE	LC 50mg/l	LC 90mg/l	Ccf	Activity
Butizin 40 SC	0.2-0.025	85.7-0.01	y = 7.48+0.11 x	<0.1000	<0.100	0.787	MT-ST
Butizin 600 SC	0.2-0.025	99.1-98.8	y = 1.98+7.92 x	0.13696	0.2004	0.868	T
Butiran 1:1	0.2-0.025	85.7-62.8	y = 6.56+0.78 x	0.01049	0.4402	0.991	MT
Icedin Super RV	0.2-0.025	2.80-0.10	y = 3.26+0.31 x	231755.3	>2317755	0.806	Sel.
Diburom 800 CE	0.2-0.025	99.3-71.4	y = 9.66+2.61 x	0.01634	0.5049	0.870	MT
Diizocab 80 CE	0.2-0.025	98.8-68.6	y = 7.75+1.42 x	0.01162	0.0921	0.876	MT
Mecloran 35 CE	0.2-0.025	80.0-71.4	y = 6.06+0.30 x	0.00035	5.6260	0.999	MT
Mecloran 48 CE	0.2-0.025	98.6-97.1	y = 7.35+0.29 x	<0.1000	0.0002	0.958	T
Oltisan M	0.2-0.025	98.6-0.01	y = 9.26+2.37 x	0.01598	0.0552	0.958	MT-ST
Oltisan Extra	0.2-0.025	98.6-82.8	y = 1.00+1.70 x	0.09995	0.9995	1.304	T
SanoltCombi400SE	0.2-0.025	98.6-64.3	y = 8.95+1.82 x	0.00671	0.0338	0.859	T-MT
2,4 SDMA 600 RV	0.2-0.025	99.8-17.1	y = 12.1+5.06 x	0.03900	0.0710	0.981	MT-ST
Banvel 600 LC	0.2-0.025	16.3-0.01	y = 7.95+5.63 x	0.29872	0.5042	0.842	ST
Zinal 500 SE	0.2-0.025	72.4-18.4	y = 7.12 +2.14 x	0.10175	0.0401	0.954	MT-ST
FRV 04 CE	0.2-0.025	62.4-0.01	y = 2.55+-3.71 x	0.22022	0.0099	0.996	MT-ST
FRV 05 LC	0.2-0.025	64.2-2.60	y = 8.00+3.87 x	0.16816	0.3597	0.980	MT-ST
FRV 06 LC	0.2-0.025	10.0-3.60	y = 4.76+1.49 x	1.43226	10.2814	0.999	ST
FRV 09 CE	0.2-0.025	77.7-6.20	y = 8.34+3.70 x	0.12449	0.2760	1.000	MT-ST
Oltest 500 CE	0.2-0.025	82.8-68.2	y = 6.15+0.28 x	0.00009	2.6255	0.980	MT
Select Super RV	0.2-0.025	65.3-28.8	y = 6.35+1.32 x	0.09431	0.8721	0.986	MT-ST
Romazin 500 SC	0.2-0.025	15.3-0.01	y =-4.24+-11.9x	0.16732	0.1306	0.995	ST
Romanex 500SC	0.2-0.025	5.71-0.01	y =-3.55+-10.7x	0.14409	0.1078	0.998	ST
Olticarb 75 CE	0.2-0.025	90.7-90.0	y = 6.23+-0.06 x	>5.5848	0.2201	0.992	T

As a result of *in vitro* testing of biological activity of 45 pesticides they could be classified in 5 categories as it can be seen in Table 5. These results confirm the previous ones (Baicu, 1982, 1987, 1990; Elad, 1991; Şesan, 1985, 1986; Şesan et al., 1998; Şesan & Oprea, 1998, 1999, 2001) and they are highly important for the IPM practices.

Conclusions

All the pesticides revealing a moderately inhibitory action to the beneficial fungus *T. viride*, used as a biocontrol agent, showed increased practical importance, being efficient in crop protection and not inducing imbalances to the agroecosystem where applied. The moderately toxic to slightly toxic pesticides and the selective ones are a very restricted group, including only 3 insecticides (Lindane 400 SC, US 1-RV, Oleoekalux + Quinalfos 3) and 13 herbicides

(Icedin Super, Banvel 600 LC, FRV 06 LC, Romazin 500 SC, Romanex 500 SC, Butizin 40 SC, Oltisan M, 1.4 SDMA 600 RV, Zinal 500 SE, FRV 04 CE, FRV 05 LC, FRV 09 CE, Select Super RV). This group of products is highly important for practice, being usable in systems of crop integrated protection without causing harm to agroecosystems.

Table 5. Classification of biological activity of pesticides on *Trichoderma viride*

Activity / Pesticides
TOXIC / *Fungicides*: Tiracarb 60 PTS, Tiracarb 600 SC, Tiramet 60 PTS, Tiramet 600 SC, FRV 12SC; *Insecto-fungicides*: Tirametox 90 PTS, Tirametox 625 SC, Supercarb 80 PSU, Supercarb T 585 SC, Procarb L, Trialin MT; *Herbicides*: Butizin 600 SC, Mecloran 48 CE, Oltisan Extra, Olticarb 75 CE;
TOXIC to MODERATELY-TOXIC / *Insecto-fungicides*:Diniconazole+Lindan, Gammavit 85 PU, Trialin; *Herbicides*: Sanolt Combi 400 SE;
MODERATELY-TOXIC / *Insecticides*: Oleoekalux+Quinalfos 3% (1998), Lindan 666 SC, Applaud + Mineral oil; *Insecto-fungicides*: Tebuconazole + Lindan, FRV 1A PTS; *Herbicides*: Butiran 1:1, Diburom 800 CE, Diizocab 80 CE, Mecloran 35 CE, Oltest 500 CE;
MODERATELY-TOXIC to SLIGHTLY TOXIC / *Insecticides*: Oleoekalux+ Quinalfos 3% (1997), US 1-RV, Lindan 400 SC; *Herbicides*: Butizin 40 SC, Oltisan M, 1.4 SDMA 600 RV, Zinal 500 SE, FRV 04 CE, FRV 05 LC, FRV 09 CE, Select Super RV;
SLIGHTLY TOXIC to SELECTIVE / *Herbicides*: Icedin Super RV, Banvel 600 LC, FRV 06 LC, Romazin 500 SC, Romanex 500 SC

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