IOBC/WPRS

Study Group "Biological Control of Fungal and Bacterial Pathogens"

and

EFPP

The European Foundation for Plant Pathology
Working Group on Biological Control

BIOLOGICAL CONTROL OF FOLIAR AND POST-HARVEST DISEASES

Proceedings of a Workshop, Wageningen, The Netherlands
29 November - 4 December 1992

Edited by
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IOBC/WPRS Bulletin
Bulletin OILB/SROP Vol.16(11) 1993

INTRODUCTION

This bulletin contains papers and summarized discussions presented at the workshop **"Biological Control of Foliar and Post-harvest Diseases"**. This workshop was organized by the IOBC/WPRS Study Group on Biological Control of Fungal and Bacterial Plant Pathogens and the Working Group on Biological Control of the European Foundation for Plant Pathology (EFPP) and held at the Wageningen International Conference Centre, from 29 November - 4 December 1992. The organizing committee consisted of C. Alabouvette, Y. Elad and N.J. Fokkema (Convener) for the IOBC/WPRS and of G. Défago, J. Hockenhull and J.M. Whipps for the EFPP. Local organizers were Y. Elad (visiting scientist IPO-DLO), J. Köhl, W.M.L. Molhoek and N.J.Fokkema of the DLO Research Institute of Plant Protection (IPO-DLO).

The reason for this first workshop in Europe on this subject is the change in public attitiude towards the use of fungicides in agriculture. National governments and the Commission of the European Community are encouraging research aimed at the reduction of the input of chemicals in the environment, as was mentioned by Mr. M. Heuver, director of the Agricultural Research Department (DLO) of the Dutch Ministry of Agriculture, Nature Management and Fisheries, in his opening address of the workshop. Biological control is nowadays considered as a generally desirable alternative to chemical disease control, including the control of leaf and post-harvest diseases which are at present controlled satisfactorily by fungicides. Whereas in the past, biological control was predominantly focussed on soil-borne diseases which are generally difficult to control with chemicals. This workshop demonstrated what plant pathologists have to offer.

Sixty participants from 15 different countries presented 57 oral presentations in the following sessions:

Strategies of biocontrol of foliar diseases
Biocontrol of post-harvest diseases
Ecology of biocontrol agents
Mode of action of biocontrol agents and biorational control
Commercialization of biocontrol agents.

The sessions were followed by general discussions focussed on the reliability of the reported biocontrol systems, the ecological constraints for optimal performance and prospects for practical apllication with respect to mass production and registration of biocontrol agents.

Thanks are due to the following organisations for supporting the workshop financially:
- The Commission of the European Communities within the framework of the RTD-programme "Agriculture and Agro-Industry including Fisheries" (AIR)
- The International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palaearctic Regional Section (IOBC/WPRS)
- The European Foundation for Plant Pathology (EFPP)
- The DLO Research Institute for Plant Protection (IPO-DLO).

Ms. H.R.M. Kentie and Ms. A. Greeven of the International Agricultural Centre and Ms. R. Straathof of the DLO Research Institute for Plant Protection (IPO-DLO) are gratefully acknowledged for their assistence in the management of the workshop.

Most of the papers in this bulletin are preliminary reports and are primarily aimed to disseminate progress in our area of research at a very early stage. Editing was done very superficially allowing efficient publication and therefore minor faults were not corrected. We thank all authors for the careful preparation of their contributions.

The organization of the workshop would not have been possible without the practical assistance of Helen van de Geijn, Carin van der Plas and Berend Verdam (St. Nicolaas). The assistence of Wilma Molhoek and Evanthia de Haan during the preparation of these proceedings was invaluable.

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Strategies of biocontrol of foliar diseases

Microbial suppression of infection

INTRODUCTORY LECTURE: MICROBIAL SUPPRESSION OF INFECTION BY FOLIAR PLANT PATHOGENS

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Summary
Competition for nutrients plays a major role in the suppression of germination, germ tube elongation, and penetration of foliar plant parts by necrotrophs; however, induced resistance and the effect of inhibitory compounds are evidenced in some pathogenic systems and when compost extracts are used. Biotrophs are generally not affected by nutrient competitors. Their infection is suppressed successfully by microorganisms that produce antibiotics or lytic enzymes or by induced resistance. Prevention of infection is incited by yeasts, filamentous fungi and bacteria; however, the mechanism responsible may vary.

Introduction

Differences in the behaviour of pathogens during the pre-penetration phase of their development on the leaf determine the way those pathogens react to the presence of other microorganisms on the leaf surface. Biotrophic pathogens, such as powdery mildews and rusts, are independent of the need for exogenous nutrients. Therefore, they can establish infection despite the presence of a nutrient-competitive microflora (Staples et al., 1962). Nevertheless, while on the leaf surface, the conidia or germ tubes of the biotrophs may be exposed to antibiotics and lytic enzymes produced by microorganisms (mainly bacteria such as *Bacillus* spp. and *Pseudomonas* spp.), that can inhibit germination and lyse germ tubes (Doherty & Peerce, 1978; Morgan, 1963). Furthermore, the germinating conidia are threatened by mycoparasites (Kranz, 1981).
Unlike biotrophs, necrotrophic pathogens, such as species of *Botrytis*, *Cochliobolus*, *Septoria*, *Cladosporium*, *Sclerotinia*, and *Alternaria*, in many cases use exogenous nutrients in order to germinate and grow on the plant surface before penetration. The necrotrophs may have to compete with the phylloplane microflora for limited nutrient sources. Reduction in the nutrient concentration generally results in a reduced rate of pathogen spore germination and slower germ tube growth, thereby reducing the number of infection courts and the extent of subsequent necrosis incited by the pathogen (Blakeman & Fokkema, 1982).
Is competition for nutrients the only mechanism responsible for microbial prevention of necrotroph infection? Study of some cases of biological control of foliar pathogens show some other mechanisms, as will be exemplified hereunder.

Studied cases of interaction

With the recognition of the importance of the presence of the saprophytic microflora on the plant surface, attempts have been made to introduce microorganisms in order to prevent infection. Numerous reports are available of attempts to control infection by means of an antagonist (see, for example, Tronsmo, 1992 and Andrews, 1992), but only a few will be reviewed here. Species of leaf bacteria (Blakeman and Brodie, 1976), yeasts (Fokkema et al., 1979), and filamentous fungi (Dubos and Bulit, 1981) can inhibit pathogens by competing for nutrients. Few bacteria are capable of

direct parasitism (Scherff, 1973). Mycoparasitism of fungi by filamentous fungi can also affect pathogens (Kranz, 1981). Antibiosis has been related to many bacterial strains (Leben and Daft, 1965), but fungi (Andrews, 1985) and yeasts (Baigent and Ogawa, 1960) have also been mentioned. Newhook (1951) and Wood (1951) inoculated senescent lettuce leaves with antagonists such as *Fusarium* sp. and *Penicillium claviforma* isolated from the same crop in order to prevent primary establishment of *Botrytis cinerea*. Fokkema (1973) showed that *Cladosporium cladosporioides* reduced the stimulatory effect of pollen on infection of rye leaves by *Cochliobolus sativus*. *Epicoccum nigrum* and *Cladosporium pullulans*, both components of the resident microflora of cabbage, reduced the incidence of successful infection of cabbage leaves by *Alternaria brassicicola*, especially if the former were pre-inoculated onto the leaves (Pace & Campbell, 1974).
Dubos et al. (1978) controlled grey mould in vineyards by *T. viride*, stressing the need to treat the vines before and after flowering in order to allow the antagonist to colonize the nutritive base of *Botrytis cinerea*. Several other researchers, who have employed *Trichoderma*-based preparations, have reported successful control of *Botrytis*. The control of grey mould on cucumber, tomato, grapes, and strawberry (Elad and Zimand, 1992) has also been attributed to successful competition for nutrients; however, other mechanisms involved in the prevention of infection are currently being studied (Zimand, Elad and Chet, unpublished). Prevention of infection by *B. cinerea* on leaves of tomato and beans by various saprophytic yeasts, bacteria, and filamentous fungi was recently attributed to competition for nutrients and to locally-induced resistance. Presence on treated leaves of inhibitory substances was minor (Elad et al., 1993-this book). A strain of *Bacillus brevis* was found by Edwards and Seddon (1992) to be effective against grey mould of chinese cabbage. In the latter system, the antagonist produced an antibiotic compound, but a reduction in the period of treated leaf wetness was noted. Both factors contributed to the reduction in *Botrytis* germination.
The effect of *Bacillus subtilis* on bean rust was shown to be very pronounced (Baker et al., 1983). Due to the production of an antibiotic, the rate of germination of uredospores was reduced and germ tubes looked abnormal. Similarly, control of geranium rust by an isolate of *B. subtilis* was due to inhibitory compounds, but competition for nutrients was probably also taking place (Rytter et al., 1989). *Erwinia herbicola* was suppressive to infection of wheat leaves by the rust fungus *Puccinia recondita*. Antibiosis was the mechanism, as an antibiotic negative mutant lacked the ability to prevent infection (Kempf and Wolf, 1989). Infection by the same pathogen and *Septoria tritici* was suppressed also by a fluorescent *Pseudomonas*, that produced two antibiotics (Levy et al., 1989). Antibiotics that leach from ascospores of *Chaetomium globosum* and diffuse on leaves inhibited infection by *Venturia ineaqualis*, but biocontrol of the disease failed (Boudereau & Andrews, 1987). Antibiosis, as well as competition for nutrients, were reported frequently for postharvest infection by necrotrophs. Recently, however, Wisniewski et al. (1991) reported the involvement of cell wall-degrading enzymes as a mode of action of the postharvest biocontrol yeast *Pichia guilliermondii* against *Botrytis cinerea*.
Pruning wounds and trunks can be protected by antagonists against pathogen invasion. When suspensions of *Trichoderma viride* conidia were applied to freshly cut trunks, the conidia were sucked into the wood vessels. The antagonist produced antibiotics while establishing, and the tree reacted by gum production. Both components prevented infection by *Stereum purpureum* (Groscloude et al., 1973).
The use of watery fermentation extracts of well-composted organic materials for the control of foliar pathogens was first suggested by the group of

Weltzien (reviewed in Weltzien, 1992 and Tränkner, 1992). By treating detached grapevine leaves with extracts of horse manure compost, they were able to control downy mildew caused by *Plasmopara viticola*. Control of diseases was later achieved in several host-pathogen systems, including *Uncinula necator* and *Pseudopeziza tracheiphila* on grape vine, *Phytophthora infestans* on potatoes and tomatoes, *Erysiphe graminis* on barley, *Erysiphe betae* on sugar beets, *Sphaerotheca fuliginea* on cucumbers, and *Botrytis cinerea* on strawberries and beans (Tränkner, 1992; Weltzien, 1992). The mechanisms suggested for the activity of the compost extracts were induced resistance and direct inhibition of the pathogens (Weltzien, 1992). *Plasmopara viticola* was also controlled by a mixture of soil, fresh cow dung and water, that was incubated for 14 days before application on vine leaves. The mechanism in this case was not induced resistance but rather direct suppression of sporangia germination (Achimu and Schlösser, 1992).
In our work, grey mould was controlled on tomato plants by compost extracts. Similarly to that work mentioned above, we were able to isolate from compost extracts bacteria that effectively controlled grey mould when applied alone; however, pasteurization of compost extracts from 14 day fermentation or filtering by Millepore membrane did not significantly nullify their ability to control grey mould. Nutrients added to the extracts with the composts did not improve their activity when tested in our system against grey mould. On the other hand, the filtration particles added to the extract at high concentration improved its biocontrol activity against *B. cinerea*. Weltzien (1992) pointed to the fact that extracts of short fermentation period lost their inhibitory effect on *B. cinerea* but "after 16 days, sterile filtrates had the same inhibitory capacity as the non filtered controls." This probably points to the effect of inhibitory compounds that accumulate in the extract during the fermentation of the compost in water; therefore, the role of the activity of microorganisms developing during the fermentation of the compost extracts should not be ruled out.
Induced resistance achieved by microorganisms or their metabolites renders the plant less susceptible to further infection by pathogens such as powdery mildew in wheat (Schönbeck and Dehne, 1986). Similar work has been done by the group of Kuc and others with cucumber and tobacco plants.
According to the literature, the involvement of mycoparasitism in prevention of infection by necrotrophs is rare.
A general rule that may be derived from our experience is that in order to be effective in the prevention of infection, biocontrol agents should be maintained at high population levels. Thus, in order to be effective, repeat applications of introduced inocula are needed, whatever the system involved. It seems that an antagonist employing variable mechanisms against plant pathogens is likely to be useful in biological control by suppression of infection.

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Control of grey mould (Botrytis cinerea) with fermented compost/water extracts.

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Summary
Fermented compost/water extracts reduce Botrytis cinerea infections. Air dried compost can be used for the fermentation process. Fermentation extracts retain their activity when freeze-dried. Bioassays with detached leaves demonstrated that the Botrytis suppresson can be enhanced to 90-100% and stabilized when protein is added to the fermentation process. An addition of protein applied as pepton rapidly increased in number spore forming bacteria in the extracts. They were largely responsible for the B. cinerea control.

Introduction

Composted organic material is characterized by dynamic microbial activity. The number of organisms and the composition of species change during the composting process (Strom, 1985; Nakasaki et al., 1985). Microorganisms isolated from compost have been shown to be potential antagonists for plant pathogenic fungi (Chung & Hoitink, 1990; Hoitink & Fahy, 1986; Kai et al., 1990). New forms of biological control of fungal plant pathogens were developed when the natural population of phyllosphere microorganisms was altered by spraying fermented compost/water extracts (CWE) onto leaf surfaces. Changing and / or stimulating the natural antiphytopathogenic potential of the phyllosphere by CWE application was successful against Botrytis cinerea, Erysiphe graminis, Sphaerotheca fuliginea, Plasmopara viticola and Pseudopeziza tracheiphila (Ketterer, 1990; Samerski & Weltzien, 1988; Stindt, 1990; Tränkner, 1992; Weltzien, 1992). The following study aimed at stabilizing the biological control agents, making them more reliable and influencing the compost/water fermentation process in such ways to make the biological control activity as independent as posssible from factors like compost composition and -age.

Methods

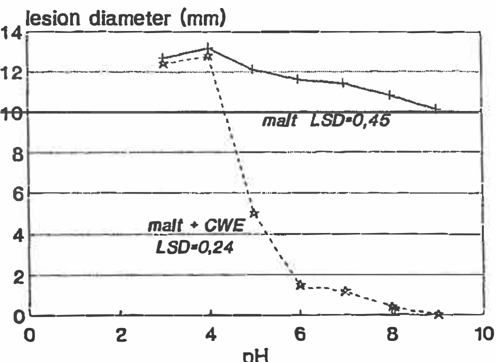
CWE was prepared by mixing compost with water 1:4 w/w. followed by 24 h fermentation at room temperature. 1 year old horse manure compost (HMC) and cattle manure compost (CMC) were used. To investigate storability, the CWE were deep frozen in liquid nitrogen and vacuum freeze-dried.
A detached leaf test based on the host-patho-system bean (Phaseolus vulgaris) - grey mold (B. cinerea) was used to determine the suppressive effect of CWE (Mansfield & Deverall, 1974). A frozen standard B. cinerea-spore suspension of 2.5×10^6 spores/ml in a solution of 11.7g/l malt extract stored at -30°C was used for inoculation. Upper surfaces of bean leaves were inoculated with 4 x 15 µl drops, containing a mixture of 5.67 parts standard spore suspension with 1 part of the extract. The efficacy of the extracts was determined by measuring B. cinerea lesion diameter. Selective media were used to determine: 1. total number of microorganisms, 2. fungi and yeasts, 3. Pseudomonas, Aeromonas species and 4. Enterobacteria.

Results and discussion

As the pH of CWE's differs, its influence in the infection droplets was investigated (fig. 1).

Figure 1:

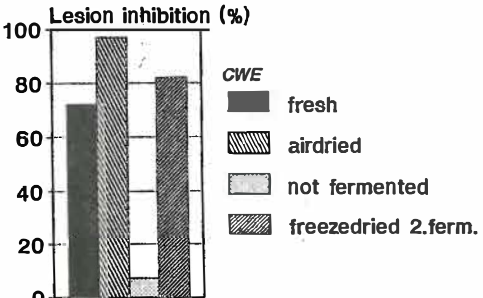
Influence of the pH on Botrytis cinerea-infection on detached bean leaves.
(malt extract = check; CWE = compost-water-extracts)



The infection was favoured by a pH ranging from 3 - 4 in pure spore suspension (control) as well as with CWE of CMC. At higher values there was a rapid decrease in the Botrytis lesion diameter after treatment with CWE but only a slight decrease in the check. For improved control with CWE on bean leaves, extracts should have a pH of 6 or above. These conditions promote many bacteria, e.g. of the genera Pseudomonas and Bacillus, which are potential antagonists of Botrytis. It was shown that pH conditions are an important and sensitive factor for successful lesion inhibition.
Most of the control effects in various host-pathogen-systems under field conditions were achieved with CWE based on composts at the age of 3-6 month (Tränkner, 1992; Weltzien, 1991). As older composts were less effective the question of storability and ways of enhancing the control effect of old composts arose. Botrytis control with CWE from air-dried composts was more effective than with CWE from fresh compost (fig. 2).

Figure 2:

Effect of different compost- or CWE-treatments on B. cinerea infection of detached bean leaves.



When the CWE was freeze dried after fermentation and fermented a second time for 4 hours before application, inhibition of Botrytis was similar to that of the CWE made on air dried compost. No effect was observed if CWE from dried compost were used unfermented. An improved Botrytis control effect was achieved by adding nutrients at the beginning of the fermentation process (fig. 3). The addition of proteins (5-7g/l) of various origin increased control of Botrytis up to 100%. If starch or suchrose were added the CWE was less effective. As the number of antagonistic bacteria in the compost decreases with the age of the compost, it is possible to promote bacteria development by adding proteins during the fementation to achieve adequate control against B. cinerea. Differences in the origin, contents and age of compost as the basic material for the preparation of CWE's can be stabilized on a high level of protective capacity for disease control by addition of proteins. Especially Pseudomonas spp. are involved in the enhanced control effect as their number was increased most by additional protein sources (tab. 1).

Figure 3:

Influence of different ways of CWE-fermentation on B. cinerea-infection of detached bean leaves. (CMC=cattle manure compost)

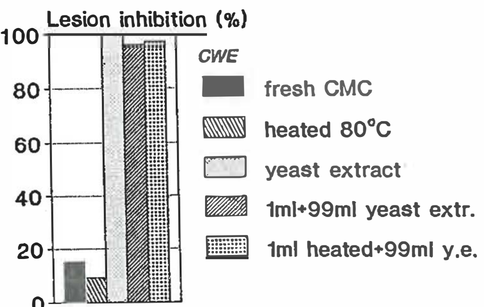


Table 1: Effect of different ways of fermentation on the quantity of special groups of microorganisms.

Fermentation	Compost	Microorganisms - c.f.u./ml			
		Total	P.s.[b]	En[c]	Fu[d]
no fermentation, fresh compost	CMC* HMC**	$5,4x10^8$ $1,2x10^8$	$< 10^4$ $3,8x10^5$	$<10^4$ $5,0x10^6$	$1,0x10^1$ $2,6x10^3$
air dried compost, fermented (24 hrs)	CMC* HMC**	$8,6x10^8$ $8,0x10^9$	$1,3x10^5$ $8,6x10^6$	$1,5x10^5$ $1,4x10^6$	0 $2,0x10^2$
fermentation+ (protein[a], 24 hrs)	CMC* HMC**	$1,3x10^{10}$ $2,6x10^{10}$	$2,0x10^7$ $4,0x10^8$	$2,0x10^6$ $2,2x10^7$	0 $2,0x10^2$

* Cattle manure compost
** Horse manure compost

[a] Yeast-extract 10g/l
[b] Pseudomonas
[c] Enterobacteriaceae
[d] Fungi

According to Phae et al. (1990) it can be assumed that also spore forming bacteria contribute to the control effect on Botrytis. CWE's had no suppressive effect if they were made with fresh material of an old compost heap or if the compost water mixture was heated for 20 minutes at 80°C before a fermentation process (fig. 3). But very active microbial antagonists against Botrytis can be found in older composts and used as an inoculum for the fermentation. For example 1 ml of CWE mixed with 99 ml yeast extract solution, fermented for 24 hours suppressed B. cinerea infection in detached leaf test. The dominant role of spore forming bacteria as antagonists against B. cinerea can also be pointed out. When only 1 ml of a heat treated CWE (80°C) was mixed with 99 ml yeast extract solution (10g/l) and left for a 24 hour fermentation the control effect remained high (fig. 3). These experiments show ways to develop fermentation and preparation methods enabling us to use also older composts and to stabilize the effect on B. cinerea. As freeze dried CWE keep their suppressive effect and the powder can be easily re-suspended and applicated, also problems of storage and standardisation seem to be solved by this treatment.
The control effect of B. cinerea by CWE with nutrient supplements was first tested in strawberries under field conditions in 1991. CMC was fermented for 6-8 days and peptone was added to stimulate bacterial growth. Strawberries were treated 4 times within the flowering stage. The infection of ripe fruits with B. cinerea was determined at 5 harvesting dates (fig. 4).

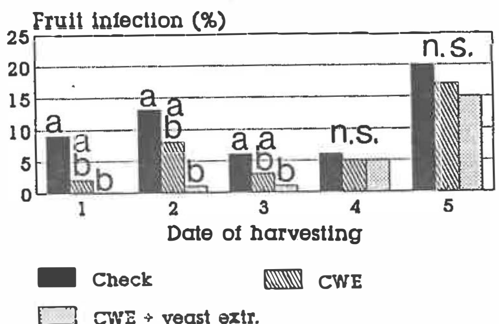


Figure 4:
Effect of different treatments on Botrytis-fruit infection of strawberries.
(CWE=compost-water-extract)

At the first three harvesting dates strawberries treated with CWE plus yeast extract showed significant reduced fruit infection. CWE's without added nutrients were less effective. Treatments with CWE's could prevent ripe fruits being infected by B. cinerea. When favourable weather conditions for Botrytis-infection occured at the end of the harvesting period, flower treatments with CWE's even ammended with nutrients could not prevent fruit infection.

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SOME EFFECTS OF WATER EXTRACTS OF COMPOST ON *BOTRYTIS CINEREA*

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Summary
Eight-day-old water extracts (CE) of a composted manure-straw mixture were assessed for antagonistic activity against *Botrytis cinerea*, using a range of tests. CE inhibited conidial germination on glass slides and reduced mycelial growth on agar. Spraying CE onto detached bean leaves two days before inoculation with droplets of *B. cinerea* conidia and mixing CE with droplets of conidia on leaves, suppressed lesion development. CE sterilised by filtration or autoclaving lost their activity completely in all tests. Weekly sprays of CE onto lettuce in the glasshouse reduced the severity of grey mould. The use of compost extract on a large-scale and its possible mode of action with respect to biocontrol are discussed.

Introduction

Grey mould, caused by *Botrytis cinerea* Pers., is an important disease of protected crops grown in the U.K. and elsewhere. Effective control of the disease has been achieved with a range of fungicides, but their widespread use has concomitantly led to the development of pathogen resistance. Further, currently recommended fungicide programmes, involving the alternate use of fungicides with different modes of action, are not always successful. Consequently, there is a need for satisfactory alternative control methods. Biocontrol using foliar spray treatments of water extracts of compost has shown some potential (Weltzien, 1992).

Materials and Methods

Production of compost and compost extract (CE)
The compost starting mixture consisted of: horse stable bedding (3,000 kg), deep litter chicken manure (150 kg), molassed brewers' grains (Sporavite; 30 kg) and gypsum (75 kg). The moisture level was adjusted to 60-70% (w/w) and ingredients were mixed mechanically. The mixture was piled in windrows in an open-sided shed and turned weekly. After *ca* 2 months, compost extract (CE) was prepared by adding compost to tap water (1:5 v/v) in an open bucket and incubating for 8 d (Weltzien, 1992). The mixture was then filtered through muslin and the filtrate (CE) used immediately. Batches of CE were also autoclaved (A-CE) at 121°C for 15 min, or filter-sterilised (FS-CE) through a 0.2 μm membrane.

Source of *Botrytis cinerea* and production of conidial suspensions
Botrytis cinerea (BC13) was originally isolated in the Netherlands from a flower of *Gerbera jamesonii* (cv. Rebecca). Conidial suspensions were obtained by flooding 8-12 d-old cultures growing on tomato leaf agar, with 0.5% (w/v) malt extract. Spore concentration was measured in a haemacytometer and adjusted to give *ca* 2×10^6 conidia ml^{-1}.

Conidial germination tests

Four treatments (CE, A-CE, FS-CE and sterile distilled water (control)) were mixed (1:1 v/v) with conidial suspension, and germination tests carried out on glass slides placed in damp chambers. Germination assessments were made after 18 h at 20°C.

Agar plate tests

Treatments as above were mixed (1:1 v/v) with 15% (w/v) tap water agar cooled to 45°, and 10 ml volumes dispensed in 9 cm diam. Petri dishes. Agar mixtures were covered with PDA (*ca* 10 ml per dish), and then inoculated centrally with a 3 mm diam. mycelial disc cut from a growing colony of BC13.

Detached *Phaseolus* bean leaf bioassays

Treatments as above were tested against BC13, using a modified bioassay of Leone & Tonneijck (1990). Eight fully-expanded detached primary leaves (cv. Groffy; 15-21 d-old) were sprayed to run-off with each treatment. Two days after spraying, leaves were inoculated by placing six 15 μl droplets of conidial suspension on the adaxial side of each leaf. After 3 d incubation at 18-20°C (14 h light/10 h dark), lesion diameters were measured and classed according to Stindt (1990). The bioassay was repeated as described, except that treatments were mixed with droplets of conidial suspension (30:70 v/v) instead of being sprayed.

Glasshouse experiment with lettuce (*Lactuca sativa*, cv. Hudson)

Immediately after planting, CE was sprayed onto four replicate plots (1.22 x 2.24 m) and then at weekly intervals. Control plots were sprayed with water. Plants were harvested six weeks later and assessed individually for grey mould, using a scale of 0-3, where 0 = no disease and 3 = severe disease (plant killed or unmarketable after trimming). A *Botrytis* severity index (0-100) was calculated for each plot:

$$\frac{1(\text{No. of plants in category 1}) + 2\,(\text{No. in 2}) + 3\,(\text{No. in 3})}{\text{No. lettuce assessed.}} \times \frac{100}{3}$$

Determination of microbial populations in CE

Actinomycetes, bacteria and fungi were determined by plating samples on water yeast agar + cycloheximide (C; 50 mgl^{-1}), nutrient agar + C (50 mgl^{-1}) and potato dextrose agar (PDA) + Aureomycin (0.32 gl^{-1}). Plates were incubated at 20°C for 3-8 d.

Table 1. Effect of different compost water extract treatments on conidial germination[a] and mycelial growth[b] of *Botrytis cinerea*.

Treatment	% germination (18 h at 20°C)	Colony diameter (mm) (72 h at 20°C)
Control (water)	70.4 (57.1)[c]	60.1
Compost extract (CE)	38.0 (37.9)	36.8
Autoclaved CE	62.1 (62.0)	60.1
Filter-sterilised CE	71.9 (58.0)	60.8
LSD (P=0.05,df=12)	(5.5)	2.9

[a]On glass slides, [b]On agar, [c]Figures in parentheses are angular transformations of % data

Table 2. Effect of different compost water extract treatments on lesion development by *Botrytis cinerea* on detached *Phaseolus* bean leaves.

Treatment	Experiment 1[a]		Experiment 2[b]	
	Lesion diam. (mm)	Lesion class (Stindt, 1990)	Lesion diam. (mm)	Lesion class (Stindt, 1990)
Control (water)	20.1	6.9	18.0	6.5
Compost extract (CE)	12.0	4.3	5.3	1.8
Autoclaved CE	19.7	7.0	19.1	6.5
Filter-sterilised CE	19.8	7.0	19.6	6.8
LSD (P=0.05,df=28)	0.8	-	1.7	-

[a]Droplets of conidial suspension applied 2 d after CE treatments
[b]CE treatments + conidial suspension (30:70 v/v) applied together in droplets

Table 3. Effect of weekly applications of compost extract on grey mould (*Botrytis cinerea*) in glasshouse-grown lettuce.

Treatment	% of diseased plants	*Botrytis* severity index (0-100)
Control (water)	97.9 a	82.8 a
Compost extract	93.8 a	60.0 b

Values in each column followed by the same letter are not significantly different using a Student's **t** test (P=0.05,df=6)

Table 4. Microbial population of an 8-d-old water extract produced from a composted manure-straw mixture.

Type of micro-organism	[a]Colony-forming units ml^{-1}
Actinomycetes	$2.4 \pm 0.1 \times 10^{5}$
Bacteria	$5.6 \pm 0.3 \times 10^{10}$
Filamentous fungi	45.5 ± 2.2
Yeasts	62.6 ± 2.8

[a]Each value is the mean of 3 replicate samples of CE ± standard error

Results and Discussion

Compost extract inhibited conidial germination on glass slides and reduced mycelial growth on agar (Table 1). Spraying extracts onto detached bean leaves 2 d before inoculation with droplets of *B. cinerea* conidia, and mixing extracts with droplets of conidia, suppressed lesion development (Table 2). However, extracts sterilised by autoclaving or filtration lost their activity completely and had no effect on conidial germination, mycelial growth, nor lesion development. This suggests that micro-organisms may suppress *B. cinerea* by both direct inhibition of spore germination and mycelial growth. Compost extract was more effective in reducing lesion development when applied with a conidial suspension as a droplet, than when sprayed onto leaves 2 d before inoculation. The reasons for this are unknown, but it may simply relate to the inability of some active micro-organisms within the compost extract to survive on the leaf surface.

Spraying compost extract onto glasshouse-grown lettuce had no effect in reducing the number of diseased plants (Table 3). However, it significantly reduced disease severity. Further work will aim to increase the efficacy of compost extract against both *B. cinerea* and other selected pathogens of protected crops.

The compost extract contained a large and varied microbial population (Table 4). The filamentous fungal population was predominantly *Penicillium chrysogenum*, *P. brevicompactum*, *Mucor hiemalis* and *Trichoderma* sp., whereas the yeast population was solely *Debaryomyces hansenii*. Interestingly, *D. hansenii* has been used successfully for biocontrol of post-harvest diseases of top fruit (McLaughlin & Wilson et al., 1992). It would be useful to consider this and other components of the CE biotic community as potential biocontrol agents.

Acknowledgements

This work was funded by the EC and MAFF. We wish to thank ir. G. Kessel (IPO-DLO, Wageningen, NL) for supplying the isolate of *B. cinerea*, and the IMI (Kew, UK) and Centraalbureau voor Schimmelcultures (Delft, NL) for filamentous fungal and yeast identification, respectively.

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BIOLOGICAL CONTROL OF *PHYTOPHTHORA INFESTANS* WITH COMPOST EXTRACTS AND SELECTED BACTERIAL ANTAGONISTS.

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Summary

A detached leaf test using potato leaves was developed for screening compost extracts (CE's) and individual microbial isolates for antagonism towards *Phytophthora infestans*. One CE (Fehnland extract) and eleven bacteria reduced *P. infestans* severity in the bio-assay consistently (50-90% reduction). The bacterial isolates C148 (*Pseudomonas fluorescens*) and B39 (*Bacillus* sp.) were the most efficient isolates selected. In two separate experiments on whole plants under controlled environmental conditions isolate C148 was able to reduce *Phytophthora* severity to very low levels (<10%).
Fehnland CE, C148 and B39 were tested in an artificially inoculated field trial. Under these outdoor conditions none of the treatments was able to affect the epidemic of *P. infestans*. However, C148 and the mixture C148 + B39 showed some potential to establish in the phyllosphere.

Introduction

Phytophthora infestans, the causal agent of potato late blight, is a serious problem in agriculture. In the Netherlands more than 50% of the total fungicide sprayings are applied against this fungus. The current concern for environment as well as human health, resulting in withdrawal of registration of several potentially harmful pesticides, has led to a need for alternative methods. One way to proceed is to develop biological control measures.
Biological control of foliar diseases is less well developed than biological control in the rhizosphere. This may be due to the adverse environmental conditions on the leaf surface and the availability of cheap and effective fungicides for the control of leaf pathogens (Andrews, 1992; Fokkema, 1991). Many experiments using bacteria as biological control agents (BCA's) against foliar plant diseases gave satisfactory results in the laboratory but failed in the field. In some cases, however, field applications of bacteria showed similar results as fungicide sprayings (Baker *et al.*, 1985; Spurr, 1982; Thomson *et al.*, 1976).
The group of Weltzien of the University of Bonn was able to control several fungal foliar diseases by spraying watery compost extracts (CE's) (Weltzien, 1991). They could reduce *P. infestans* severity to acceptable levels in six different field experiments over 3 years by spraying CE amended with a mixture of seven micro-organisms.
At IPO-DLO several compost extracts and more than 200 micro-organisms, isolated from compost extract or from the phyllosphere of field-grown potatoes, were screened for potential suppression of *P. infestans* in a detached leaf test. The most effective bacterial isolate was tested on whole plants in two separate experiments, whereas one CE and the two most effective bacterial isolates and several of their combinations were tested in a field experiment.

Material and methods

Compost extracts (CE's) were prepared by mixing compost and water (1:9 w/w). The extracts were incubated for 14 days (the so-called extraction time) at 20°C. During this fermentation process microbial populations build up to levels between 10^7 and 10^8 colony forming unit's (CFU's) per ml. Extracts were filtered and applied undiluted to individual leaves or to whole plants to test their potential to suppress *P. infestans*.
Individual bacteria, cultured for 48 hours in Trypticase Soy Broth (TSB) at 24°C, were applied undiluted to detached leaves or to whole plants to test their ability to suppress *P. infestans*.
The detached leaf test consisted of twelve composite potato leaves which were placed in a plastic tray, lined with a sheet of wetted filter paper on the bottom, to obtain a high humidity within the tray. The petioles of the leaves were fitted into pieces of wetted oasis (Floral Foam on ureumformaldehyde basis). Each CE or bacterium was tested several times and each test consisted of two trays, half of the leaves of each tray were sprayed with the undiluted suspension by means of a pressure air sprayer until run-off, the rest of the leaves were sprayed with tap water. After one day incubation at room temperature, all leaves were sprayed with a sporangial suspension (10.000 sporangia ml^{-1}) of *P. infestans.* After 5-7 days the percentage sporulating leaf area could be assessed.
Antagonism on whole plants was tested in controlled climate chambers, 16 hr photoperiod, day- and night temperature 18°C and 15°C, and 75-85% relative humidity (RH). Five-week-old plants were sprayed with undiluted bacterial suspensions or with tap water with a PMT Tamson air sprayer until run-off. After 24 hours incubation all plants were sprayed with *P. infestans* (20.000 sporangia ml^{-1}), just before the dark period started, and covered with plastic bags to create optimal conditions for *Phytophthora* infection. The next morning the covers were removed. The first symptoms appeared 5-7 days after inoculation and after 12 days every leaf of each plant was assessed for percentage sporulating/necrotic leaf area.
A field trial with two potato cultivars (Santé and Agria) and six treatments (Fehnland CE, Fehnland CE amended with C148 and B39, C148, B39, a tankmix of C148 and B39 and a water control) was carried out in four replicates (a total of 48 plots). Each plot consisted of 100 plants. The trial was designed as a randomized block experiment per cultivar and barley served as an isolation crop to minimize interplot interference. Seven sprayings were done at weekly intervals in the evening at a rate of 1000 liter ha^{-1}.
Samples, consisting of ten leaflets per plot, were taken randomly twice a week (half crop height), one day before spraying and three days after spraying. The samples were shaken in 1/4 strength Ringer solution with 0.1% Tween 80 for one hour and leaf washings were plated on 1/2 strength Trypticase Soy Agar (TSA) for determination of total numbers of bacteria per cm^{-2}. Countings were done by means of an Image Analyzer. Furthermore, disease assessments were made twice a week to follow the *Phytophthora* epidemic. Weather data, like temperature, relative humidity and leaf wetness, were recorded during the whole experiment.

Results

About 200 bacteria, 20 yeasts and several compost extracts were tested in the bio-assay, the results are summarized in table 1. None of the yeasts showed antagonistic activity against *P. infestans*, eleven bacterial isolates showed a consistent reduction of *P. infestans* severity. A fluorescent *Pseudomonas* (C148) and a *Bacillus* sp. (B39) appeared to be the best candidates and were chosen for further investigation on suppression of *P. infestans*. Most of the compost extracts did not affect *Phytophthora* infection, only Fehnland reduced *P. infestans* consistently

in the detached leaf test.

Table 1: Summary results of screening on detached leaf test.

Isolate	N	% Reduction of sporulation	Compost	N	% Reduction of sporulation
C8	6*	68 ± 19**	GFT	4	3 ± 1
C35	3	63 ± 16	Bavaria	2	20 ± 1
C40	3	62 ± 25	Champost	5	31 ± 16
C119	5	57 ± 24	Fresh Ch.	2	31 ± 18
C130	5	56 ± 14	Fehnland	5	67 ± 6
C148	11	77 ± 9			
C155	6	63 ± 12			
C161	4	54 ± 23			
F14	3	65 ± 14			
F17	3	66 ± 16			
B39	4	72 ± 19			

Isolates originate from compost extract (C- and B- isolates) or from the potato phyllosphere (F-isolates). GFT, Champost and Fresh Ch. are standardized Dutch composts whereas Bavaria and Fehnland are commercially produced in Germany (* number of replicate experiments, ** standard deviation.)

Until now only one bacterial isolate (C148) has been tested on whole plants under controlled environmental conditions. In two separate experiments this isolate reduced *P. infestans* severity to very low levels (fig 1).

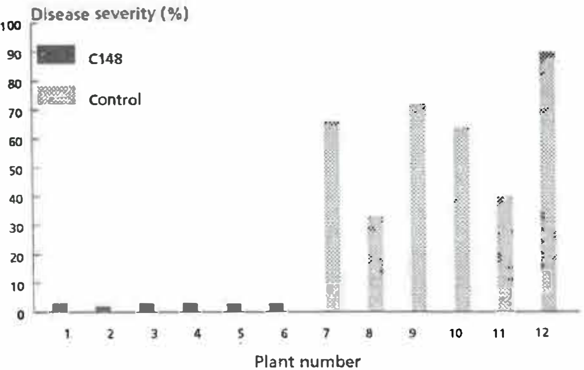

Fig 1: Control of *Phytophthora infestans* with C148 on whole plants.

In the field trial none of the applied treatments was able to reduce *P. infestans* severity. However, the applied bacteria showed some potential to establish in the phyllosphere. Especially on leaves treated with C148, the total number of bacteria per cm^2 was significantly higher than on leaves which were not treated with bacterial cultures (Fig. 2). Although not very likely, this effect might be due to the inflow of nutrients (TSB) to the phyllosphere together with the applied bacteria.

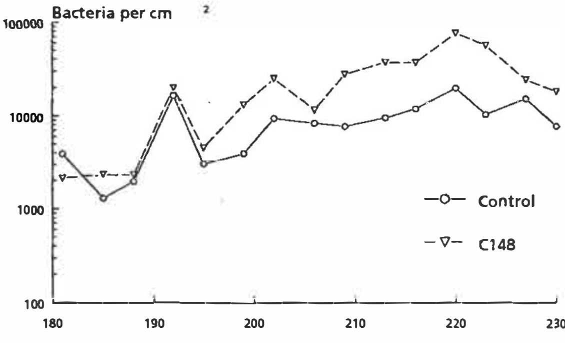

Fig 2: Bacterial densities in the phyllosphere of field grown potato leaves of the cultivar santé.

Discussion

Biological control of *P. infestans* has not been established until now except for the work of the group of Weltzien who were able to control this fungus under field conditions with amended compost extracts. Infection by *P. infestans* takes place when the spores are located on the leaf surface in the presence of free water. Spores start to germinate and within 4 hours the fungus may penetrate the leaves. The biological control agent should be active and present in high enough population densities in this particular period if a secondary metabolite is involved. If induced resistance is involved the population build up does not need to take place during this interaction period. The biotrophic character of *P. infestans* excludes competition as possible mechanism. The discrepancy between the results of laboratory experiments and field trials may be attributed to the adverse environmental conditions on the phyllosphere in the field situation, like low relative humidity, high temperatures and UV-radiation, preventing the build up of a large enough antagonistic population. Under field conditions the bacteria were probably not able to survive or multiply as well as under lab conditions.

Efficacy and reliability of antagonistic field applications may be improved by a better understanding of the mechanism(s) involved. At present experiments are done combining mechanistic studies and population dynamics in order to reveal processes responsible for the antagonistic action of C148 and B39 and to improve their efficacy and reliability.

Acknowledgement

Part of the research was financed by the European Commission (CAMAR).

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EFFECTIVENESS OF WATERY COMPOST EXTRACTS AGAINST ALTERNARIA ALTERNATA IN CUCUMBER

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Summary
Pathogenic strains of the fungus Alternaria alternata can be severely damaging to cucumber crops in plastic greenhouses in Crete during the winter. Watery compost extracts were tested in the laboratory against this pathogen with respect to spore germination and infection of detached leaves and young cucumber plants. Data obtained indicated that spore germination was strongly inhibited by the extracts and infection of detached leaves was reduced to nearly 100%. Young cucumber plants were protected from infection by 30 to 50% depending on the compost.

Introduction

The fungus Alternaria alternata causes severe losses in cucumber grown in plastic greenhouses in Crete during the winter months when climatic conditions are similar to those favourable to grey mould. Control by fungicides further increases the already high chemical input in this crop. Recently successful control of grey mould and several other foliar diseases by watery compost extract has been reported by Weltzien (1992). Based on this data it was considered that biological control agents associated with watery compost extract were worth testing against A.alternata. Results of these experiments are reported here.

Materials and Methods

The composts tested were made after fermentation of the following organic material; a. straw, b. cow manure, c. sheep manure, d. chicken manure, e. a 1:1 mixture of chicken manure and seaweed. A compost of German origin was also included. If it is not otherwise stated all composts were amended with peptone (0.5%) and dextrose (1%).

Each compost was mixed with water (1/8 w/v dilution) and placed in an orbital incubator at a temperature of 22°C for 2 days at 200 rpm. At the end of the second day the mixture was filtered through cheese cloth and the filtrates used the same day.

1. Spore germination tests on cucumber leaf discs.
Detached cucumber discs of 16 mm diameter were placed in Petri dishes. The filtrate of each type of compost was mixed with an equal volume of a spore suspension of A.alternata containing 10^6 spores/ml in 1% malt extract. One drop of the aliquot was placed on each leaf disc and incubated in a growth chamber at 28°C for two hours. Afterwards prints of the spores were taken using collodion and their germination was recorded using microscopy.

2. Infection of detached cucumber leaves.
Leaves of Dutch-type cucumbers were inoculated by placing 10 drops of the above filtrate and spore suspension (50 µl containing 10^6 spores/ml) on the adaxial surface of the leaves. 1% malt extract was used as the nutrient source for infection. In the screening tests the drops consisted of 25 µl of malt extract

and spore suspension and 25 µl of compost extract. Infections were recorded after 6 to 7 days incubation at 19°C by the method used by Stindt (1990) for Botrytis. In an additional test fermented compost was filtered using single or double layered cheese cloth, centrifuged to remove particulate matter, or sterilised either by steam or filtration.

3. Infection of young cucumber plants
Experiments were conducted on young cucumber plants with two fully developed leaves. Ten punctures were made with a hot needle on each leaf. The composts were sprayed on the same day as the artificial inoculation with A.alternata (1.10^6 spores/ml). After an incubation for 5 days at 27-28°C records of infection were taken on a 0 to 5 visual rating scale (0 = no infection, 5 = expansion zone > 1 cm diameter).

Results and Discussion

Spore germination of A.alternata was inhibited by 80 to 98% depending on the type of compost and the experiment. It can be seen that the cattle compost gave the best control with the least control obtained from straw compost but these results varied between experiments. Nevertheless experiments always exceeded 80% inhibition of spore germination. Composts were also effective in protecting detached cucumber leaves from infection. Again at least 85% protection was observed and with some composts no infection occurred (Table 1.). Reduced effectiveness was achieved when tests were made on young cucumber plants. Less than 50% protection was achieved in these experiments and with some extracts protection as low as 20% was observed (Table 2.).

Table 1. Effect of several compost extracts on spore germination of A.alternata and the infection of detached cucumber leaves.

Composts	% Spore Germ.	% Infection
Straw	18.0 b	7.1 b
Cattle	2.0 c	2.4 b
Sheep	6.0 bc	0.9 b
Chicken	8.7 bc	0.5 b
Chicken + Seaweed	6.7 bc	5.2 b
German	3.4 c	0.0 b
Control	85.4 a	50.5 a

Table 2. Effectiveness (% protection) of composts against A.alternata infection on young cucumber plants.

Composts	% Protection	
	1st Exper. (28°C)	2nd Exper. (19°C)
Cattle	30.5 a	42.8 a
Sheep	20.8 a	48.7 a
Chicken	19.0 a	37.4 a
Chicken + Seaweed	24.0 a	29.7 a

The results obtained indicated that watery compost extracts were highly effective against spore germination of A.alternata on cucumber leaf discs. They were also very effective in reducing infection of detached cucumber leaves. When applied to young cucumber plants their effectiveness was reduced. There is some evidence that at 19°C extracts protected young cucumber plants better than at 28°C (Table 2.). No consistent difference was observed between the effectiveness of the different composts.

Steam and to a greater extent filter sterilised compost extracts retained some of their inhibitory activity indicating that as well as the presence of the microorganisms there may be metabolic products present within the extract. The difference between the steam and filter sterilised results would suggest the presence of a mixture of heat sensitive and resistant substances.

Table 3. Treatment of compost and effectiveness (% protection) against A.alternata infection. Results are the mean of four composts.

Treatments	% Protection
Filtration through cheese cloth	88.4 bcd
Double Filtration	69.3 bc
Steam Sterilisation (125°C, 20 min.)	11.4 a
Filter Sterilisation	33.1 b
Centrifugation	72.9 bc

Acknowledgement
The authors are grateful to Dr.B.Seddon and Mr.S.G.Edwards of the University of Aberdeen for correcting this manuscript.

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A NEW SYSTEM FOR BIOLOGICAL WHEAT BUNT CONTROL (TILLETIA CARIES) BY SEED TREATMENT WITH NUTRIENT CARRIERS.

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Summary
Wheat bunt (*Tilletia caries*) remains one of the most destructive wheat diseases if not regularly controlled by fungicidal seed treatment. As its use should be limited for environmental concerns, or is generally rejected by organic growers, we have searched for alternative control methods. Seed treatment with biologically active compost extracts reduced the infection rate under greenhouse conditions, but failed outdoors. Amendment of the extracts with nutrient carriers such as wheat flour and skim milk powder increased the efficiency significantly. Seed incrustations with nutrient carriers alone were highly effective. Commercial seed coating is a possible control method. A *Bacillus subtilis* strain was found to be a good bio-control agent. It produces the antibiotic Iturin. The seed coating method is now tested on a wider scale and is expected to be suitable for organic and environmental farming systems.

Introduction

In Germany wheat bunt was successfully controlled through seed treatment with mercury containing compounds since 1913, until these compounds were banned for toxicological reasons in 1983. Since then organic fungicides are used. Their application as dust was banned in 1990 and replaced by slurry application. As no other control methods were available, organic growers refusing to use synthetic chemicals, were confronted with increasing *Tilletia* problems, which they could only overcome with the expensive and risky method of hot water treatment or by buying certified, hopefully *Tilletia*-free seed from conventional producers. This fact and growing concern about the introduction of chemical pesticides into the food chain through birds or soil inhabiting organisms caused us to look for biological control mechanisms (Becker, 1992). As *Tilletia* chlamydospore germination is not subject to soil fungistasis (Weltzien 1963) but can be suppressed by antagonists, we tried the use of microbially active complex substrates such as composts and of nutrient carriers as microbial stimulants.

Results

At first lyophylized watery compost extracts were used for seed incrustation at a rate of 100g/kg and tested on *Tilletia* contaminated seeds (3 g spores/-kg, ca. 90.000 spores/seed) under greenhouse conditions. This resulted in reduced infections from 48 % in the check to 13 % after use of horsemanure compost, an efficiency rate of 73 %. When we tried to verify these results under field condition, we failed to achieve any significant reduction in two different locations. The same was true if watery extracts were used for seed dipping before planting.

In a second approach we tried to follow up an earlier report on *Tilletia* suppression by nutrients. A first test included wheat flour, skim milk powder and ground algae (*Laminaria saccharina*) in their effect on spore germination. They all suppressed the germination completely if added at a rate of 10 % to the medium.

If these treatments were tested under field conditions in 1988/89, the results were most encouraging. Control effects between 89 and 97 % were achieved based on an infection rate of 36 % in the untreated check. Skim milk powder proved to be the most effective compound. However, the rather high rate of nutrients applied (400g/kg seeds) caused problems in mechanical seeding and in some cases reduced the rate of emergence.

In 1989/90 we therefore used a seed coating technique. Water and compost extracts were used as the binding liquid. The results confirmed the findings from the year before, with skim milk powder again, being most effective, even at a reduced rate of application (300 g/kg). Still the emergence rate was negatively affected in most cases, though compost extract as binding liquid counteracted this negativ effect. Also the yield was not significantly suppressed by the reduced plant density. To reduce the risk of plant losses, a dosage test with skim milk powder followed in 1990/91 now using the commerial seed coating technique by the SUED company. It showed that an amount of 160g/kg seed was fully sufficient to suppress the *Tilletia* infection almost completely.

The microbiological analysis of the treated seed surface revealed a high frequency of suppressive bacteria, with spore forming Bacilli being most frequent and active. Isolation experiments resulted in a single strain with high *Tilletia* suppressive characteristics. The most promising strain was a *Bacillus subtilis* culture, producing the antibiotic Iturin as active substance.

Discussion

The field experiments proved, that biological suppression of *Tilletia caries* on winter wheat is possible through application of nutrient carriers such as wheat flower and skim milk powder. They stimulate the multiplication of antagonistic bacteria on the seed surface and prevent the *Tilletia* spores from germinating. Highly effective *Bacillus* strains can be isolated und used as biocontrol agents. Considering the difficulties of getting microorganisms registered as bio-pesticides, we prefer to recommend seed coating with nutrients rather than pure culture application. Seed coating can be performed commercially and applied to foundation seed to get a *Tilletia* free stand. From this, untreated seed may be used until *Tilletia* attack increases again. Another strategy would be to treat all seed annually with on farm equipment such as concrete mixers or tumbling drums, to keep the *Tilletia* infection rate always below the economic threshold level. Wide spread experimentation with this new method under different climatic conditions is encouraged. It should be noticed, however, that soil borne infections can probably not be controlled by this system.

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BIOLOGICAL CONTROL OF THE PEACH TWIG PATHOGEN MONILINIA LAXA.

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Summary
The brown rot fungus *Monilinia laxa* is an important pathogen in the peach-growing areas of Spain where causes severe twig blight. Biological control offers the possibility of reducing deficiencies of chemical control. A programme to develop a biological control method in order to introduce it in an integrated management control system was begun and attempts are discussed in this report.

The brown rot fungus *Monilinia laxa* is an important pathogen in the peach-growing areas of Spain where causes severe twig blight during May and June under certain conditions. This blight impairs fruiting potential in the year of the epidemic and in the following year. Twig blight is usually controlled by several fungicide applications during the growing season, or by removing the diseased shoots. The evolution of tolerance in *M. laxa* to certain fungicides, the increasing cost of chemical control, and the threat of regulatory restrictions all point to the need for alternative control methods (Zehr, 1982).

Biological control offers the possibility of reducing such inconveniences. However few attempts have been made in this direction. We began a programme to develop a biological control method in order to introduce it in an integrated management control system.

We began our approach to the biological control of *Monilinia laxa* by screening antagonists among the fungal microflora of peach twigs.

Samples of healthy peach twigs were taken at random from an orchard in Madrid, Spain, every other month, for one and a half years. Fungi were isolated from samples and numbers determined. The fungi isolated were mostly Hyphomycetes (species of *Alternaria, Penicillium, Cladosporium, Botrytis, Cercosporidium, Epicoccum, Gliomastix, Nigrospora, Paecilomyces, Fusarium, Trichoderma*, etc), although Coelomycetes (species of *Phoma, Cytospora* and *Gloeosporium*), and fungi belonging to Zygomycotina (*Mucor* and *Rhizopus*) and Ascomycotina (*Nitschkea, Sordaria, Elsinoe* and *Sclerotinia*) were also present.

Nineteen fungi were consistently isolated from twigs throughout the year and were grouped in three classes:(i) fungi with maximal populations early in the growing season (*Aspergillus candidus, A. flavus, A. fumigatus, A. niger, A. terreus, Botrytis cinerea, Epicoccum nigrum, Mucor pusillus, Penicillium chrysogenum, P. frequentans, P. islandicum, P. purpurogenum, P. raistrickii* and *Rhizopus stolonifer*), (ii) fungi with maximal populations in summer (*Alternaria alternata, A. tenuissima, Cladosporium herbarum* and *C. sphaerospermum*) and (iii) fungi with a maximum in the late growing season and in winter (*Gliomastix murorum*). Numbers of these species varied in twigs throughout the year suggesting an active growth and reproduction in twigs (Melgarejo *et al*, 1985). These fungi may be considered as resident in twigs according to the criteria of Leben (1965).

Once the basic study on the microflora of peach twigs was done, we studied the antagonistic relationships among the different isolated fungi and *M. laxa*. Since resident antagonists may have more potential for biological control than casual ones we have limited our search for antagonists against *M. laxa* to the 19 resident species. Antagonism against *M. laxa* by these fungi was evaluated in dual cultures in different culture media. Five fungi (*Aspergillus flavus, Epicoccum nigrum, Penicillium chrysogenum, P. frequentans and P. purpurogenum*) out of the 19 tested showed antagonistic properties against *M. laxa in vitro* (Melgarejo *et al*, 1984) and were further tested *in vivo* tests under laboratory conditions. Cuttings of peach twigs were treated with a mycelial plug of each of the antagonist before being inoculated with a plug of the pathogen. After 24 days of incubation, the extent of

colonization of *M. laxa* in the twigs was determined. No recovery of *M. laxa* from the cuttings treated with *P. purpurogenum* and *P. frequentans* was observed (Melgarejo *et al*, 1984). *A. flavus* and *E. nigrum* also inhibited the extent of pathogen colonization. These results showed that four out of the five fungi tested interfere with the pathogenic capacity of *M. laxa in vivo*.

In later experiments we tested the potential for biocontrol of *M. laxa* of these four fungi (*A. flavus, P. frequentans, P.purpurogenum,* and *E. nigrum*) in the field. Experiments were done in peach orchards located in Zaragoza (Spain) in spring and autumn. On each tree twigs chosen at random were first inoculated with *M. laxa* and 10 days later were treated with one of the 4 antagonistic fungi. Other twigs were first treated with the antagonist and 10 days later inoculated with *M. laxa*. The extent of colonization of *M. laxa* on the twig tissues was determined 35 days after the inoculation with *M. laxa*. The introduction of the antagonists before the pathogen in the spring experiment resulted in a significant reduction in the growth of the pathogen in tissues (accounted 35% for *E. nigrum*, 21% for *A. flavus*, and 41% for *P. purpurogenum* and *P. frequentans* over control) (Melgarejo *et al*, 1986). The autumn experiment was less informative because of the slow growth of the pathogen, but protection was evident when *E. nigrum*, *P. purpurogenum* and *P. frequentans* were applied. Our results clearly indicated the potential for disease reduction of *M. laxa* in twigs by these four fungi, particularly by *E. nigrum* and both *Penicillium* spp. Moreover, the fact that these fungi are residents in peach trees enhances this potential. However, *A. flavus* was discarded for further experiments because it induced lesions in fruits.

These results stimulated further work in order to define the conditions for the practical use of these antagonists in a biological control strategy. Experiments were carried out in experimental and commercial orchards located in Zaragoza and Madrid (Spain) from 1986 to 1992 with various preparations of spores and/or mycelium of the antagonists *P. frequentans, P. purpurogenum* and *E. nigrum*. Hereafter we present the results obtained with each antagonist.

Successful biological control of twig blight induced by *M. laxa* with *P. frequentans* isolate 909 depends on establishing antagonist populations higher than 10^3 colony forming units/g fresh twig weight (De Cal *et al*, 1990). Only preparations of spores and/or mycelium of the antagonist containing nutrients (wheat bran, malt and yeast extracts or nutrient agar) gave significant reductions in disease severity (from 38 to 80% over control), which were comparable to that given by the fungicide captan. Populations were consistently higher on those shoots receiving these preparations of *P. frequentans:* antagonist populations in these treatments ranged from 10^5-10^6 cfu/g fresh twig weight 20 days after application of treatments to 10^4-10^5 cfu/gr fresh twig weight after 36 days more. These populations were consistently higher to those mantained in other treatments.

A study on the effects of a pesticide schedule (based on applications of the fungicides captan, dinocap and benomyl and the insecticide methomyl) on the epiphytic fungi of peach twigs carried out throughout 2 years showed that the most abundant fungi on both untreated and treated twigs were *Alternaria* spp., *Cladosporium* spp. and *Penicillium* spp. Populations of *Cladosporium* spp. and *Alternaria* spp. were considerably depressed by the pesticides. However, numbers of *Penicillium* spp. were less depressed (especially *P. frequentans*). *Penicillium* spp. (especially *P. frequentans*) were more frequent on treated twigs than *Alternaria* spp. and *Cladosporium* spp. from the end of May to August (De Cal & Melgarejo, in press). These results show that *P. frequentans* readily colonizes twigs and is highly competitive with other components of the mycoflora of peach twigs, which makes it a good candidate for biological control.

All of these results suggest that the competence for nutrients and space could play an important role in the biocontrol. However, we can also postulate other modes of action such as antibiosis. *P. frequentans* isolate 909 produces two active substances against *M. laxa*, which showed significant inhibition of the pathogen on peach twigs. (De Cal *et al*, 1988).

Biocontrol obtained after application of *E. nigrum* isolate 282 was variable each year, depending on the relative disease severity during the first two-three weeks after infection, the climatic conditions and the

nutrients added to inoculum of *E. nigrum*. In 1988 and 1989, treatments with *E. nigrum* conidia+mycelium and with *E. nigrum* conidia+mycelium+nutrients gave significant reductions in disease severity (from 50 to 85%) over control, comparable to that given with captan (Table 1). In contrast, in 1991 the addition of appropriate nutrients to the *E. nigrum* inoculum (enhancing the growth and sporulation of *E. nigrum* but not of *M. laxa*) was necessary to obtain successful control (Table 1).

Table 1: Effects of *E. nigrum* applied in various forms and captan on the colonization (mm) of peach twigs by *M. laxa*[a].

Treatment[b]	Years		
	1988	1989	1991
C+M	-	25 ± 4 bc	48 ± 5 a
C+M+N_1	8 ± 3 b	32 ± 5 b	-
C+M+N_2	-	-	30 ± 6 b
Captan	10 ± 3 b	21 ± 5 c	34 ± 5 ab
No treatment	58 ± 8 a	64 ± 5 a	40 ± 5 a

[a] Evaluated 50 days after the inoculation with *M. laxa*. Data are the means of 42 observations ± standard error of the mean. Means followed by the same letter in a column were not significantly different ($p \leq 0.05$) by Wilcoxon non-parametric range tests.
[b] C+M: conidia+mycelium; C+M+N1:conidia+mycelium+nutrient solution 1 (1%malt extract, 0.3% yeast extract and 0.1% Tween 80); C+M+N2: Conidia+mycelium+nutrient solution 2 (2% lactose, 1% KNO_3 and 0.06% Nu-Film); captan 1.3 g a.i./l.

A compound with antibiotic activity towards *M. laxa* was isolated from 10-day-old potato-dextrose stationary cultures of isolate 282 of *E. nigrum* (Madrigal *et al*, 1991). The ultraviolet, infrared, proton nuclear magnetic resonance and mass spectra of the purified antibiotic indicated that it was flavipin. Flavipin was active against *M. laxa* and the application of cell-free liquid cultures of *E. nigrum* to peach blossoms previously inoculated with *M. laxa* prevented blossom and twig infection, as did spraying with propagules of the fungus. Although evidence of the role of antibiosis in the biocontrol of *M. laxa* by *E. nigrum* needs further work, these results suggest that flavipin may have a role in it. Campbell (1956) related the strong suppression of the pathogenic activity of *Helminthosporium sativum* by *E. nigrum* to the production of toxic compounds.

Isolate 828 of *P.purpurogenum* produces lytic enzymes which are directly implicated in the degradation of fungal cell walls, such as β-1,3-glucanase, β-1,3,(4)-glucanase, β-1,6-glucanase, polymethyl-galacturonase and chitinase (Larena & Melgarejo,in press). *P. purpurogenum* directly attacked the mycelium of *M. laxa* when both fungi were grown together (Melgarejo & M.-Sagasta, 1986). The enzymatic complex of *P. purpurogenum* also attacked the mycelium of *M. laxa* lysing its hyphae and spores, resulting in complete destruction of the mycelium. Antagonism, responsible for some types of biological control, may operate by exolysis, breaking down the walls of fungal pathogens (Campbell, 1989). Various examples of biological control have been related to the activity of hydrolytic enzymes (Ordentlich *et al*, 1988). We also obtained biocontrol of *M. laxa* with *P. purpurogenum* in peach twigs. Biocontrol obtained with this antagonist was variable each year depending on the climatic conditions. In years with favourable climatic conditions for the development of the antagonist reductions of disease severity reached values of 80% over control (as in 1988 and 1989), comparable to that given with captan. However,

no reduction of disease severity was obtained (as occurred in 1990 and 1991) under unfavourable climatic conditions.

A further reduction in the severity of twig blight may be possible by increasing the concentration of inoculum of each antagonist in sprays, by improving the formulation of antagonist inoculum, by using isolates better adapted to the phyllosphere than the ones used in these experiments, and by applying mixtures of antagonists. Also, a better knowledge of the ecological milieu where the antagonists are being introduced and the knowledge of the mechanisms of biocontrol could lead to improve it.

Bollen (1982) pointed out that differences between pathogen and antagonist sensitivity to fungicides should be considered in the development of new integrated control strategies. *M. laxa* is about 20 times more sensitive to captan than *E. nigrum*, 8 times more sensitive to captan than *P. frequentans* and 5 and 3 times more sensitive to iprodione and vinclozoline, respectively, than *P.purpurogenum*. Alternating treatments of antagonists and fungicides could be a possibility to improve the control of *M. laxa*. Integrated treatments (biological and chemical) may have additional advantages over the application of antagonists or chemicals alone: the total fungicide application is reduced and the chemical acts as a safeguard for those years in which the weather conditions do not favour the activity of the antagonists. The possibility of exploiting other isolates of the studied species, which are more resistant to fungicides needs also to be examined.

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A MINIATURISED *IN VIVO* ASSAY FOR THE SCREENING OF POTENTIAL ANTAGONISTS OF *BOTRYTIS CINEREA* ON TOMATO PLANTS.

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Summary

In order to screen large numbers of micro-organisms for their potential as biocontrol agents for the protection of tomato plants against infection by *Botrytis cinerea*, an assay was developed on tomato leaf disks (2 cm in diameter) kept on wet filter paper in 14.5 cm diameter Petri dishes. The filter papers were placed on water-soaked pads to maintain relative humidity within the dishes close to saturation throughout the test. The plates where incubated in a growth chamber at 23°C (14 hrs daylight) and 18°C (10 hrs darkness). The test involved 2 steps: The leaf disks were (i) spray-inoculated with a water-suspension of the potential antagonist after a superficial burn had been effected in the center, and (ii), 24 hours later, challenge-inoculated with *B. cinerea* delivered as a 5 µl drop of a 10^7 cells/ml suspension. In a control treatment, the suspension of potential antagonist was replaced with sterile water. Disease development was monitored over time and recorded as percent decayed surface area.

Introduction

In screening micro-organisms for their potential antagonism to plant pathogens it is often desirable to use *in vivo* techniques to avoid eliminating organisms with modes of action other than antibiosis. Our objective was to develop an *in vivo* assay to find micro-organisms able to protect tomato plants from infections by *Botrytis cinerea*. A test based on whole tomato plants would be difficult to implement for the screening of large numbers of micro-organisms, given the size of tomato plants and the time required to grow them. The present paper reports the development of a quantitative disease bioassay on tomato leaf disks

Methods

The plant material consisted of leaf disks (2 cm in diameter) cut with a cork borer from the 4th and 5th leaves of tomato plants, *cv.* Monalbo, at the 8-10 leaf stage. The disks were placed in sets of 8 on wet filter paper. In order to maintain relative humidity around the disks near saturation for the duration of the test, the paper carrying the sets of leaf disks was placed on top of water-soaked pads inside 14.5 cm diameter Petri dishes. The plates where incubated in a growth chamber at 23°C (14 hrs daylight) and 18°C (10 hrs darkness).

The inoculation procedure involved 2 steps: a spray-inoculation of the disks with a water-suspension of the potential antagonist (*ca.* 10^6 cells/ml for fungi and yeasts and *ca.* 10^7 cells/ml for bacteria) followed, 24 hrs later, by a challenge-inoculation with *B. cinerea* at the center of the disks. In sets of control disks, the suspension of potential antagonist was replaced with sterile water. In order to facilitate the assessment of antagonistic activity, our objective was to obtain rates of infection consistently near 100% on the control treatments. Various ways of applying wounds on the leaf disks and various

inoculum levels and glucose or phosphate amendments (Leone & Tonneijk, 1990) were tested for their effectiveness in facilitating infection by *B. cinerea*.

The resulting disease development was quantified as the diameter of the lesion or the percent of disk area colonized by the fungus.

Results & discussion

The most difficult step in the development of the assay was obtaining successful infection of the leaf disks after inoculation with *B. cinerea* alone. Infection was not observed at all or was irregular on disks left intact or puncture-wounded immediately before inoculation. On tomato cultivars *Monalbo* and *Rondello*, results were similar whether dry or wet (5 µl drops) spores were used and for inoculum concentrations of 10, 100, 1000 or 10000 spores/drop.

An infection rate of nearly 100% was regularly obtained if a superficial burn was effected with a red-heated pin head (1.5 mm in diameter) in the center of the disks just before inoculation with 5 µl of a 10^7 spores/ml suspension (Figure 1). Infection rates decreased sharply with inoculum densities below 10^7 spores/ml (Table 1).

If sterile water was sprayed onto the disks immediately after burning and inoculation with *B. cinerea* was done 24 hours after burning the disks, as required for the control disks, low infection rates were again observed (Table 2). Rinsing the leaf disks for 3 hours before utilization in the assay or amending the spray water with 50 ppm each of penicillin, streptomycin and chloramphenicol failed to improve the rates of infection on disks inoculated 24 hours after burning (Table 3). Amendments with antibiotics even decreased the development of infection on disks inoculated immediately after burning.

Figure 1:

Progress of infection of tomato leaf disks branded with a red-hot pin head just before inoculation with a spore suspension (5 µl, 10^7 cells/ml) of *Botrytis cinerea*.

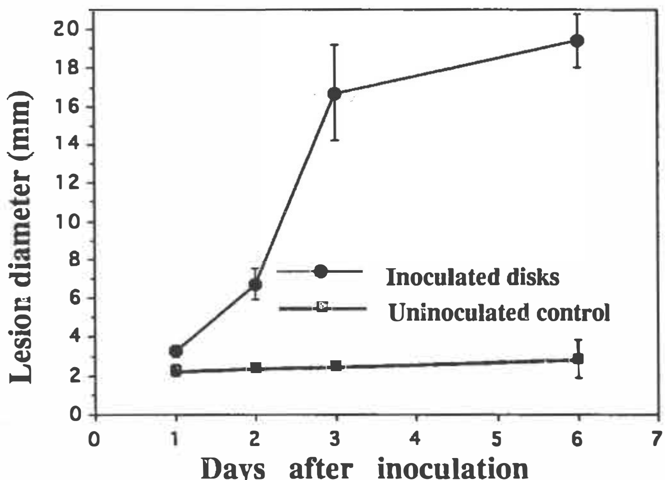


Table 1: Effect of inoculum density of *Botrytis cinerea* on the infection of tomato leaf disks. A superficial burn was effected in the center of each disk with a red-hot 1.5 mm diameter pin head immediately before inoculation.

	inoculum density (spores / ml)				
	10^3	10^4	10^5	10^6	10^7
% infected disks	0	20 ± 13	0	33 ± 21	83 ± 11
average lesion diameter (mm)	3.4 ± 0.4	4.2 ± 1.1	3.5 ± 0.5	7.6 ± 3.5	15.6 ± 2.7

*each value is the average for 8 replicate disks 5 days after inoculation

Amending the inoculum suspension with 0.1 M glucose, 0.07 M phosphate or glucose+phosphate significantly increased the rate of infection on disks wounded 24 hours before inoculation (Table 4). Phosphate was the most efficient in facilitating infection. However, phosphate and glucose+phosphate amendments significantly reduced the rate of infection on disks inoculated immediately after wounding (Table 4).

Table 2: Effect of a delay between wounding and inoculation on the infection of tomato leaf disks by *B. cinerea*. Wounding consisted of a superficial burn applied with a red-hot 1.5 mm diameter pin head.

Disease rating at 4 days after inoculation	Time of inoculation	
	24 hrs after wounding	immediately after wounding
% disks infected	43.8* ± 3.6	93.8 ± 3.6
% leaf disk area colonized	17.3 ± 3.0	86.4 ± 6.9

*each value is the average for 4 sets of 8 leaf disks ± standard error

Table 3: Effect of two pre- and post-wounding treatments on the infection of tomato leaf disks by *Botrytis cinerea*. Wounding consisted of a superficial burn applied with a red-hot 1.5 mm diameter pin head.

	Treatment of leaf disks prior to wounding					
	Rinse 3 hrs in sterile water			No rinse		
	post-wound spray with			post-wound spray with		
Time of inoculation	sterile water	anti-biotics	no spray	sterile water	anti-biotics	no spray
immediately after wounding	16.0*	12.9	17.2	15.9	8.5	12.9
24 hours after wounding	4.7	4.9	8.1	6.2	4.0	9.5

*each value is the average lesion diameter (in mm) for 8 replicate disks 4 days after inoculation

Table 4: Percent area colonized by *Botrytis cinerea* on tomato leaf disks 4 days after inoculation with 5 µl of a suspension containing 10^7 spores/ml

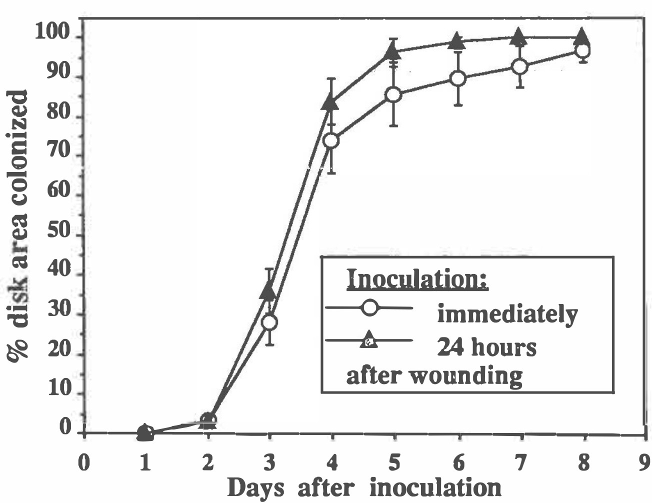
	Type of amendment in inoculum suspension			
Type of wound on the leaf disks	no amendment	glucose[1]	phosphate[2]	glucose[1] + Phosphate[2]
no wound	0*	18.1 ± 9.4	0	0
burn 24 hours before inoculation	1.3 ± 1.3	51.3 ± 16.4	91.3 ± 5.2	66.3 ± 15.7
burn immediately before inoculation	92.5 ± 3.1	96.3 ± 2.6	65.6 ± 13.2	75.0 ± 8.2

*each value is the average for 8 replicates ± standard error
[1]glucose at 0.1 M
[2]KH_2PO_4 at 0.07 M

To keep the assay as close as possible to natural conditions of infection, we sought an alternative to amending the inoculum with nutrients. A burn of larger diameter was effected with the head of a nail (3 mm diameter). This procedure resulted in successful infection in 100% of the trials, whether inoculation occurred immediately after wounding or 24 hours later in the presence of a water spray (Figure 2), and it was adopted for the test of antagonism in our study.

Figure 2:

Progress of infection of tomato leaf disks branded with a red-hot pin head just before inoculation with a spore suspension (5 µl, 10^7 cells/ml) of *Botrytis cinerea*.



In the conditions of the present study, the wounded but uninoculated leaf disks remained alive and appeared healthy for the duration of our observations (up to 12 days). The tissue surrounding the wound seemed to defend itself against infection, and successful colonization by *B. cinerea* was not consistently obtained unless a sufficiently large area was burned in the center of the disks. One might speculate that this was necessary for the pathogen to "gather strength" by growing saprophytically on dead tissue before it faced the defence reactions of the host. While this complicated somewhat the development of the assay, the presence of live plant tissue may also be seen as an advantage for the utilization of the test to screen potential biocontrol agents: It may avoid discarding micro-organisms that interfere with pathogenesis rather than with saprophytic development of *B. cinerea*. (Such micro-organisms, for example, would act as stimulants of the host's defence mechanisms). It may also allow to screen out micro-organisms that are not only antagonistic to *B. cinerea* but also pathogenic to tomato.

This bioassay was used to screen a collection of microorganisms and several have been selected for their ability to protect leaf disks from infection by *B. cinerea*. Validation of the bisoassay will be possible by testing those microorganisms on whole plants.

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CONTROL OF INFECTION AND SPORULATION OF *BOTRYTIS CINEREA* ON BEAN AND TOMATO LEAVES BY YEASTS AND OTHER SAPROPHYTIC MICROORGANISMS

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Summary
Saprophytic microorganisms (bacteria, yeasts or filamentous fungi) were screened for their ability to reduce severity of grey mould (*Botrytis cinerea*). Isolates of the yeasts *Rhodotorula glutinis* and *Cryptococcus albidus*, of the bacteria *Xanthomonas maltophilia*, *Bacillus pumilus*, *Lactobacillus* sp. and *Pseudomonas* sp., and of *Gliocladium catenulatum* were found to control grey mould in bean and tomato plants. Their ability to reduce germination of conidia and severity of rot symptoms on detached leaves and to control the disease on whole plants, under controlled conditions was good, consistent and at least similar to the activity of the known biocontrol agent *Trichoderma harzianum* T39 (non-formulated). The pathogen was applied at a rate of 10^5-10^6 conidia/ml. The selected saprophytes were effective in control at concentrations 10^6-10^7 cells/ml. The yeast isolates were tested at low nutrient level and found effective. All isolates compete with the germinating conidia of *B. cinerea* but resistance induced in the host by live or dead cells may also explain their activity of some of them. Inhibitory compounds were detected rarely on treated leaves. Establishment of yeast populations on healthy and *Botrytis*-infected leaves and on flowers was tested. Reduction of sporulation of the *Botrytis* after its establishment was tested with the aim of reducing inoculum pressure in the crop. Most of the above mentioned isolates and isolates of saprophytic fungi reduced sporulation of the pathogen. The final objective of this study is to combine infection control with sporulation control in order to reduce grey mould epidemics.

Introduction
On the plant surface, nutrients are necessary for the growth of saprophytes and pathogens that have an epiphytic growth phase before penetration (Blakeman, 1972). Yeasts, bacteria, and filamentous fungi are common inhabitants of plant surfaces (Blakeman, 1982; Fokkema, 1971; Dickinson & Wallace, 1976). The importance of nutrient competition in antagonism of necrotrophic fungi have been demonstrated (Brodie & Blakeman, 1975). Yeasts and bacteria are known for their ability to reduce conidial germination by competition for nutrients (Blakeman & Fokkema, 1982; Dik et al., 1991), however, the introduction of antagonists into the phyllosphere to control leaf infections by necrotrophic pathogens has been in many cases only moderately effective. In order to provide effective control, the introduced microorganism has to be established in the phyllosphere before the pathogen arrives. Once infection has occurred it may be ineffective.
The purpose of the present work was to study the biocontrol of grey mould by different saprophytes in order to prevent infection. Furthermore, inhibition of pathogen sporulation on was tested in order to reduce production of inoculum of the pathogen that endangers the developing crop.

Results and discussion
Screening of antagonists was carried out with isolates of saprophytes belonging to the following genera (all from the Ecology collection at the IPO-DLO Wageningen, The Netherlands):
Bacteria: *Bacillus, Lactobacillus, Pseudomonas, Aeromonas, and Xanthomonas.*
Yeasts: *Rhodotorula, Cryptococcus*, and some unidentified white and pink isolates.
Filamentous fungi: *Trichoderma, Gliocladium, Alternaria, Penicillium, Cladosporium, Chaetomium, Sesquicillium, Arthrinium, Ulocladium, Chaetomium, and Aureobasidium.*

The following tests were run 3-8 times on plant material of bean (*Phaseolus vulgaris* L. Cv. Groffy) and tomato (*Lycopersicon esculentum* Mill. Cv. Money Maker):
- Germination of conidia of *Botrytis cinerea* in water drops on detached leaves (incubated for 24h).
- Estimation of germ tube length of germinated conidia (after 24h).
- Calculation of germ tube biomass produced by the pathogen on leaves.
- Severity of symptoms induced by the pathogen on leaves (4d after infection).
- Grey mould of whole plants (after 14d), of flowers (after 4d) or of pods (after 7d) - in growth room.

- Sporulation of the pathogen on lesions produced after infection of leaves (live and dead).

Treatments with the antagonists were carried out with inoculation - for the infection tests - and after establishment of the pathogen - for sporulation tests.

The following isolates (with isolate number) were found more effective: The yeasts *Rhodotorula glutinis* (F147) and *Cryptococcus albidus* (F131 and 053), the bacteria *Xanthomonas maltophilia* (B39), *Bacillus pumilus* (F17) *Lactobacillus* sp. and *Pseudomonas* sp. (C155), and the fungus *Gliocladium catenulatum* (162). The biocontrol agent, *Trichoderma harzianum* T39 from Israel was used as a standard throughout the study. A summary of indicative results is given in Table 1 (for bean) and in Table 2 (for tomato).

Table 1: Effect of saprophytic microorganisms on *Botrytis*[1] infection of bean

Isolate (10^6/ml)	Germination of *Botrytis* conidia (%)	Germ tube biomass[2]	Symptom severity on detached leaves[3]	Flower infection (%)	Pod infection[3]	Whole plant grey mould[3]
Control	86	2600	3.3	100	4.1	3.3
F147	0	0	0.5	66	0.8	0.6
F131	25	105	1.7	51	0.7	1.0
053	5	10	0.7	50	0.9	1.4
F17	7	21	0.8	0	0.7	0.4
B39	22	88	0.3	17	1.4	0.2
C155	7	30	0.02	21	0.7	0.3
Lacto	15	95	1.1	26	0.3	0.9
162	0	0	0.5	0	0.7	0.35
T39	8	90	0.4	26	1.1	0

[1] Conidia of *B. cinerea* were applied at a rate of 10^5/ml.
[2] Multiplication of the number of germinated conidia out of ten by the length of germ tube.
[3] Severity index of 6 degrees where 0=healthy plant material.

Table 2: Effect of saprophytic microorganisms on *Botrytis*[1] infection of tomato

Isolate (10^6/ml)	Germination of *Botrytis* conidia	Germ tube biomass[2]	Symptom severity on detached leaves[3]	Whole plant grey mould[3]
Control	80	1200	3.4	2.3
F147	0	0	0.6	0.4
F131	9	60	1.2	0.65
053	0	0	1.23	0.35
F17	22	240	1.6	0.6
B39	0	0	0.65	0.9
C155	5	10	1.22	1.5
Lacto	6	24	1.05	1.6
162	4	8	1.5	1.5
T39	5.2	41	1.1	0.15

[1] Conidia of *B. cinerea* were applied at a rate of 10^5/ml.
[2] Multiplication of number of germinated conidia out of ten by the length of germ tubes.
[3] Severity index of 6 degrees where 0=healthy plant material.

As is shown in Tables 1 and 2, the selected microorganisms indeed reduced the germination of conidia of the pathogen on leaves of bean and tomato. Moreover, the severity of the disease incited by the pathogen was reduced by these isolates down to a satisfactory level.

The level of nutrients affected the ability of the biocontrol agents to reduce germination and to control the disease. However, the involvement of mechanisms other than competition for nutrients are involved in the interaction between the antagonists and the pathogen on the plant surface. Therefore, the following tests were carried out on bean leaves:

- Elimination of phylloplane microflora by surface disinfection.
- Extraction of water soluble inhibitory compounds from leaf surfaces treated by antagonists.
- Application of antagonists at sites remote from the infection site.
- Application of dead cells of antagonists.

Results are summarized in the following table.

Table 3: Possible involvement of general microflora, inhibitory products and induced resistance mechanisms in the suppression of *Botrytis* infection by potential biocontrol agents

Isolate	Effective disease control on surface disinfected leaves	Presence of inhibitory substances on leaves	Control by dead cells	Remote effect on same leaf
F147	+	+-	+-	+
F131	-	+-	+-	+
053	-	+-	+	-
F17	++	-	+	-
B39	++	-	+-	-
C155	+	+-	+	+
162	-	-	+	+
T39	++	-	+-	-

Table 3 demonstrate involvement of general leaf microflora (1st column) and induced resistance (3rd and 4th columns) but not inhibitory compounds in the interaction of some of the biocontrol agents with the system of *Botrytis* infected plant. It can be assumed that mechanisms other then competition for nutrients may be involved in the control of grey mould by the tested microorganisms. However, the relative importance of any particular mechanism is unclear.

Yeast cells which were applied on the leaves and flowers of the test plants resulted in population of 10^3-10^4 cells per cm^2 of leaf. Higher populations were detected in necrotic tissues. Disease control was achieved even at low nutrition regime on the leaves.

Suppression of sporulation was tested on dead plant leaves or stem segments of bean or tomato. Plant material was killed by autoclaving, exposure to microwave or gamma irradiations. Further, saprophyte microorganisms were applied to lesions caused by *B. cinerea* on leaves attached to, or detached from, the plants. Suppression of sporulation was achieved by fungal isolates of *Penicillium* sp. (023), *T. viride* (T048), *Ar. montagnei* (242), *Al. alternata* (317,319), *U. atrum* (385), *Ar. phaeosporum* (243), *G. catenulatum* (017), and *C. globosum* (256). As various saprophytic microorganisms were found able to reduce infection and/or sporulation of *B. cinerea*, it is possible that they could reduce epidemic development in crops susceptible to the pathogen. However, a proper formulation of the biocontrol agents is needed in order to allow their performance under unfavourable field conditions.

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Acknowledgements: The financial support of the International Agricultural Centre (IAC) at Wageningen and of The Europene Environmental Research Organization (EERO) to one of us (Y.E.) is gratefully acknowledged.

ANALYSIS OF AND STRATEGIES FOR THE BIOCONTROL OF BOTRYTIS CINEREA BY BACILLUS BREVIS ON PROTECTED CHINESE CABBAGE

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Summary
Monitoring of antibiosis by dual culture incubation *in vitro* showed strong antagonism of *Bacillus brevis* and its antibiotic, gramicidin S to *Botrytis cinerea.* This antibiosis was less effective *in planta* with Chinese cabbage where higher levels of *B.brevis* spores or pure antibiotic were necessary for antagonism. *In situ* analysis of the system using protected Chinese cabbage grown under polythene and spray treatment with *B.brevis* spore preparations gave successful biocontrol exceeding that expected from *in planta* observations with antibiosis as the mode of antagonism. Methods for the analysis of microbial interactions *in planta* and *in situ* were developed and a second mode of antagonism, based on accelerated drying of the leaf surface, is considered to be important *in situ* in addition to antibiosis.

Introduction

To achieve biocontrol in field situations and to work towards the commercial exploitation of these systems for agricultural purposes careful choice has to be made concerning the pathogen, the BCA (biological control agent) and the crop. The criteria on which decisions are made are crucial to the success of these systems and it is important that at an early stage of the work thought is given towards all aspects of development from initial *in vitro* testing of the system through to *in planta* studies, field trials (*in situ* studies) and then to final formulation and application. It is frustration itself to have developed a successful antagonist that gives biocontrol in laboratory studies which is then not amenable to methods of packaging, distribution, reconstitution, spraying etc. These points must be taken on board at the outset. If possible it is best to carry out experiments *in situ* or at least under conditions that match field situations but this is not always possible for a variety of reasons. It is not possible to standardise procedures and conditions in field situations where variability of environmental parameters (temperature, humidity, etc.) is a characteristic feature of the system. The best that can be achieved is with the use of stored and protected crops where some degree of control is possible even though fluctuations in environmental parameters still occur. It is not easy to perform biochemical and physiological tests in field situations on the interactions of pathogen, host and BCA. There is a lack of methodology and techniques available to obtain this information, with the result that little is known about the detailed interactive ecophysiology of pathogen and BCA under these conditions. It is obvious that this area of research requires to be opened up if biocontrol systems are ever to become adopted.

Choice of Biocontrol System

Botrytis cinerea is a serious pathogen world-wide of numerous important crops and is listed by the fungicide industry among the top six major global diseases (Jutsum, 1988). When fungicides are used in an attempt to control *B.cinerea* the organism invariably becomes fungicide-resistant (Locke & Fletcher, 1988).

Breeding programmes for host resistance are not considered a feasible option because of the non-specialised nature of the pathogen. The spread and dissemination of the pathogen and its infection stages and characteristics with respect to susceptible hosts are generally well documented (Coley-Smith et al., 1980) and this information is useful when targeting BCA's for its control. For these reasons B.cinerea is considered an appropriate choice of organism for biocontrol studies. The BCA Bacillus brevis is a well studied and characterised bacterium and its biology, life-cycle, biochemistry, physiology and genetics (especially of antibiotic production) are well documented (Kleinkauf & von Dohren, 1982). Interests in antibiotic production and function have largely been the driving force behind these studies but interests in sporulation and spore characteristics have also yielded detailed information that can be used for its application as a BCA (Nandi et al., 1985). B.brevis Nagano produces a single well-documented antibiotic gramicidin S which we have shown to be antifungal to B.cinerea (Murray et al., 1986). Gramicidin S-negative mutants of B.brevis Nagano are also available (Iwaki et al., 1972). The organism is a spore-former and can survive in the environment and the fact that it is a prokaryote makes it compatible for integrated use with fungicides directed against eukaryotes. Finally, it is amenable to genetic engineering if necessary (Mittenhuber et al., 1990). These attributes make B.brevis a strong candidate for consideration as a BCA against B.cinerea. Chinese cabbage (Brassica campestris L spp. pekinensis var. Granaat) is a host plant for B.cinerea where the pathogen causes grey mould disease. In Britain Chinese cabbage is grown as a protected crop under glass or polythene during Spring and Autumn. Such crops experience damp, cool conditions which are conducive to B.cinerea and provide suitable features for the study of grey mould disease and its biocontrol. Therefore this system is a careful choice for the investigation and development of methodologies and strategies involved.

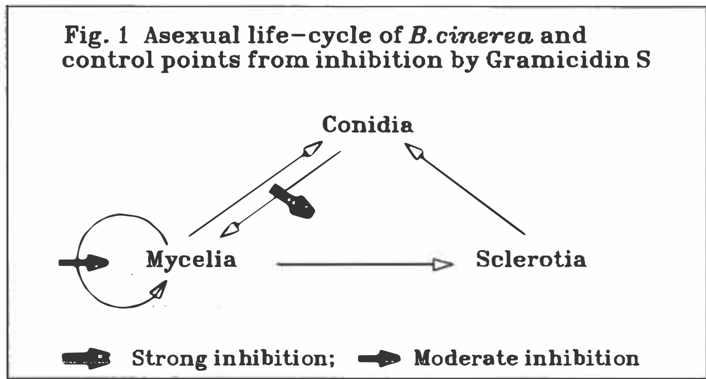


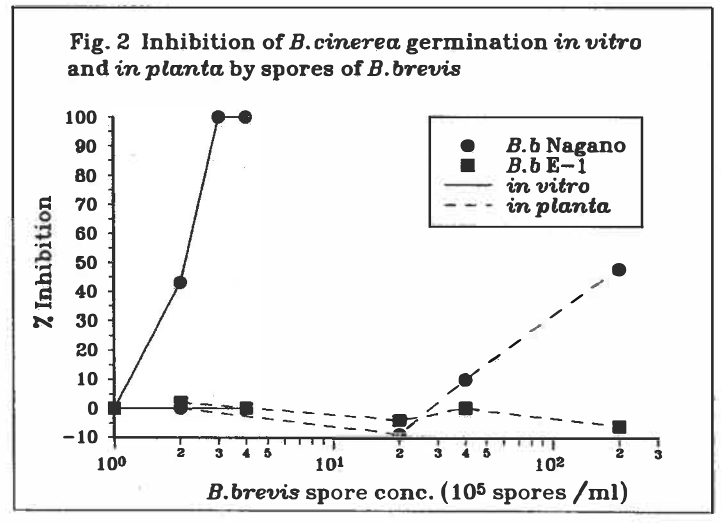
Fig. 1 Asexual life–cycle of *B.cinerea* and control points from inhibition by Gramicidin S

Results and discussion

In vitro studies using gramicidin S and B.brevis spores (which naturally carry gramicidin S on their outer surface) showed that both mycelial growth and conidial germination of B.cinerea were inhibited by the antibiotic. A consideration of the simplified asexual life-cycle of B.cinerea (Fig. 1) indicated that inhibition at either of these points in the life-cycle could provide strategies for disease control. However, germination of conidia was 10-

fold more sensitive to inhibition than mycelial growth with complete inhibition by gramicidin S at 5 µM (equivalent to 1-2 x 10^5 spores/ml). Since spread and initiation of disease is via conidia alighting on the crop then inhibition of germination of conidia provides an ideal target point for biocontrol. Experiments with gramicidin S-negative mutants of B.brevis indicated that these spores did not inhibit germination and confirmed antibiosis as the mode of antagonism in vitro.

In planta studies were made using leaves of Chinese cabbage sprayed to run-off with gramicidin S or spores of B.brevis. Leaf sections inoculated with conidia of B.cinerea when incubated at 15°C and 100% relative humidity showed inhibition of conidial germination only at much higher concentrations of gramicidin S (500 µM) and B.brevis spores (2 x 10^7 spores/ml). Again gramicidin S-negative mutant spores did not inhibit (Fig. 2). In order to monitor germination of conidia initial microscopic studies were made by fixing and staining with phenolic Rose Bengal. Such studies were prone to inaccuracy since conidia could be lost from the leaf surface during the staining procedure. The use of vital staining of conidia with Calcofluor M2R prior to observation gave best results since no staining procedure was required after experimentation. The differences between in vitro and in planta sensitivity is thought to be due to gramicidin S binding to the leaf surface and thus reducing the levels available to interact with B.cinerea.



When studies were carried out in situ using polythene tunnels planted with Chinese cabbage under conditions otherwise conducive to grey mould infection successful biocontrol was achieved using B.brevis spore preparations. Disease control was as effective as the standard fungicide treatment (Iprodione and B.brevis gave 70 and 68% reduction in disease index respectively). During these studies methods were developed for the specific retrieval of B.brevis and B.cinerea from the environment (Edwards & Seddon, 1991a,b) and their levels were monitored during these field trials as were temperature, relative humidity and leaf surface wetness. The interesting observation was made that treatment with B.brevis spore preparations led to an accelerated drying of the leaf surface.

It is thought that this phenomenon of minimising periods of leaf wetness could act together with antibiosis in reducing germination and infection of B.cinerea on the leaf surface. In this way B.brevis, with two modes of antagonism, may prove to be an ideal BCA for B.cinerea.

This system might also be applicable to other plant pathogens. Those pathogens where leaf wetness is necessary for spore germination and infection could be targeted by the B.brevis system. Both foliar crops or stored vegetables/fruits might be targeted with successful results. Future investigation will no doubt confirm or refute these suggestions.

Acknowledgement
This work was supported by AFRC grants PGI/PEI/506.

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Strategies of biocontrol of foliar diseases

Microbial suppression of dissemination of necrotrophs

INTRODUCTION: MICROBIAL SUPPRESSION OF DISSEMINATION OF NECROTROPHS

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Different strategies for biocontrol of foliar diseases may be approached. In accordance with the three major stages in the life cycle of the pathogen we may distinguish between: microbial suppression of infection, sporulation and survival of the pathogen. Biological control agents should not only interact with the most vulnerable stage of the pathogen but also with stages allowing a long interaction period between the antagonist and the pathogen. Biocontrol based on interference with the dissemination of the pathogen allows a much longer period of interaction than biocontrol aimed at the prevention of infection, where the pathogen may escape from antagonism in the phyllosphere by penetrating the leaf. This possible advantage of interactions aimed at the dissemination of the pathogen is already fully recognized in the biocontrol of biotrophic pathogens which is largely based on the reduction of sporulation capacity by mycoparasites. In analogy with this, it is worth to investigate the impact of suppression of sporulation of necrotrophs on disease development. Sporulation suppression of necrotrophs implies interactions in dead plant tissue. This could be either necrotic lesions caused by the pathogen itself or dead leaves and plant debris serving as a substrate for the pathogen. While biotrophs are mainly antagonized by specific mycoparasites, colonization and subsequent sporulation of necrotrophs may predomantly be affected by saprophytes competing for the same substrate.

Although relatively new as a biocontrol strategy, suppression of sporulation by saprophytic fungi is a well-known natural phenomenon. Cook (1970) observed that *Fusarium culmorum* sporulated abundantly on straw of wheat sprayed with fungicides and consequently deprived of naturally occurring saprophytes, but not on "weathered" straw naturally colonized by saprophytes.
In the early seventies, control of scab in apple and pear (*Venturia* spp.) was attempted by spraying urea just before leaf fall (Burchill and Cook, 1971; Margraf et al. 1972; Latorre and Marin, 1982). This resulted in a reduction of the ascospore production on leaf litter in the following spring by about 90%. This reduction of primary inoculum delays the onset of the epidemic, but since secondary conidial infections are not affected, the impact on disease development is temporarily and only measurable if urea is applied on a large scale.
The mechanism seems to be based on the rapid decomposition of the leaf litter and the stimulation of a general antagonistic microflora which interferes with the development of the pseudothecia.
Direct application of selected antagonists, such as *Chaetomium globosum* and *Athelia bombacina*, to apple leaf litter was also very effective in suppression of the ascospore formation of the hemibiotrophic *Venturia inaequalis* (Heye and Andrews, 1983; Young and Andrews, 1990; Miedtke and Kennel, 1990). This demonstrates that antagonism between saprophytes and the pathogen in dead leaf tissue is potentially an efficient means to interfere with the dissemination of the pathogen. For the scab control these treatments need to be integrated in other control measures directly affecting the conidial infections.

A detailed study of the wheat microbial community of wheat straw revealed that the basidiomycete *Limonomyces roseipellis* suppressed the pseudothecia development of *Pyrenophora tritici-repentis*, the causal agent of tan spot (Pfender, 1988; Pfender and Wootke, 1988). Particularly in no-tillage farming practises, surface-borne residues are an important source of inoculum (Zhang and Pfender, 1992). Treatment of straw residues in the field with *L. roseipellis* reduced the inoculum by 60 to 80%. This was however, not sufficient for the control of the disease (Pfender et al. 1993). Again the general principle of interaction in dead leaves was clearly demonstrated, but overall control failed, also because conidial production and infection was not affected.

Therefore, biological control should address all stages of dissemination. Biles and Hill (1988) found that *Trichoderma harzianum* could reduce sporulation of *Cochliobolus sativus* in lesions. Trutmann et al. (1982) were the first who demonstrated that treatment of aerial plant parts with the mycoparasite *Coniothyrium minitans* reduced the viability of sclerotia of *Sclerotinia sclerotiorum*.

The latest achievements in this area of research will be presented in this chapter.

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BIOSUPPRESSION OF INOCULUM PRODUCTION BY BOTRYTIS CINEREA IN STRAWBERRY LEAVES.

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Summary

Gliocladium roseum, Penicillium sp. and Trichoderma viride suppressed sporulation potential of B. cinerea by 97-100% in attached strawberry leaves in the greenhouse, by 58, 64, and 48%, respectively, in overwintered leaves in the field, and by 89-100%, 59-100%, and 53-87% in green leaves in the field. Biosuppression increased with temperature from 10 to 25 C. Only G. roseum was highly suppressive at 10 and 15 C. Evidence is presented to support the concept that the antagonists suppressed B. cinerea when the pathogen invaded senescent or dead leaf tissues, and that competition between the antagonists and the pathogen for leaf substrate was a key mechanism of biocontrol.

Introduction

Key strategies for managing grey mold fruit rot of strawberry, caused by Botrytis cinerea, are suppression of conidial production of the pathogen in inoculum sources, and protection of the flowers against infection by conidia of the fungus (Sutton 1990). The principal source of B. cinerea in Ontario strawberry fields is mycelium in dead strawberry leaves. Conidia dispersed from the leaves may infect the flowers, which are the main pathway of infection of the fruits (Sutton 1990). Fungicides are widely used for protecting the flowers and fruits, and chlorothalonil effectively suppresses inoculum production of B. cinerea in the dead leaves (Sutton 1990). However fungicide tolerance of B. cinerea is widespread, and fungicide use is strongly opposed by the public because of possible residues in strawberries and the environment. Recently, biological methods were developed as alternatives to fungicides for protecting the flowers and fruits against the pathogen (Peng and Sutton 1991). In these studies, several fungal antagonists isolated from strawberry also were observed to suppress B. cinerea in strawberry leaves used in screening tests, however the potential for biocontrol in the leaves remains unexplored (Sutton and Peng 1992).

In examining biocontrol of B. cinerea in the leaves it is important to consider relationships of the pathogen with the leaf tissues. Strawberry leaves are highly receptive to infection at the bud stage and when expanding but receptivity declines at later stages (Sutton 1990). The fungus remains quiescent in the epidermal cells until the leaves senesce, after which it may grow and sporulate.

In the present study, antagonists selected in the earlier screening work (Peng and Sutton 1991) were evaluated for effectiveness in suppressing growth and sporulation potential of B. cinerea in strawberry leaves in the greenhouse and field. Temperature effects, and interactions of the antagonists with the pathogen were investigated under controlled conditions.

Biocontrol tests.

The fungal antagonists Fusarium sp., Gliocladium roseum, Myrothecium verrucaria, Penicillium sp., Rhodotorula glutinis, and Trichoderma viride were compared with chlorothalonil 50 F (1 ml product/100 ml water) for effectiveness in suppressing B. cinerea in strawberry leaves. Except in one field test, leaves were inoculated with conidial suspensions of B. cinerea containing 10^5 or 10^6 spores/ml in the

field and greenhouse, respectively. The antagonists were applied as aqueous suspensions containing 10^7 spores or cells/ml water plus surfactant (50 μl Triton XR/100 ml water). In the greenhouse, attached strawberry leaves were inoculated with B. cinerea, and kept in a humidity chamber for 24 h at 20-21 C and then in a greenhouse at 20-30 C for 2 wk. The leaves were then sprayed with inocula of the antagonists, chlorothalonil, and water plus surfactant, and kept in the humidity chamber for 24 h and in the greenhouse for 2 days. Discs were then cut from the leaves and incubated on paraquat-chloramphenicol agar (PCA) for 7 days, after which conidiophores of B. cinerea on the discs were counted.

In the field plots in 1989, the six antagonists, chlorothalonil, and water plus surfactant were applied to overwintered leaves on 26 May and 3 June. These leaves were not artificially inoculated with B. cinerea. In a second study, young leaves produced in spring were inoculated with B. cinerea on 13 May and the treatments were applied on 13 and 20 June. Leaves in the two studies were harvested on 10 and 27 June, respectively, and discs cut from the leaves were placed on PCA for estimation of grey mold incidence. Similar studies were conducted in 1990 using three of the antagonists (G. roseum, T. viride, and Penicillium sp.) applied to individual green leaves or inundatively to whole plots. Sampled leaflets were quartered and kept in humidity chambers for estimation of sporulation incidence of B. cinerea.

Temperature effects.

To examine temperature in relation to biocontrol, leaf discs were washed in sterile water, inoculated with B. cinerea (10^6 spores/ml), and incubated in high humidity at 20-23 C for 48 h. The three antagonists (10^7 conidia/ml), chlorothalonil, and water plus surfactant were then applied to the discs, which were kept on PCA at 10, 15, 20 or 25 C for 7 days prior to estimation of conidiophores of B. cinerea.

Temperature was investigated also in relation to conidial germination and germ tube elongation of the three antagonists on leaf discs. The discs were inoculated, placed on PCA at the four temperatures, sampled at intervals, fixed, stained, and examined microscopically. Percent germination and germ tube length of the antagonists were estimated on the disc samples.

Effects of leaf senescence and death.

Leaf discs inoculated with B. cinerea (10^6 conidia/ml) were incubated in high humidity for 24, 48, 72 and 96 h and then kept on PCA for 72, 48, 24 or 0 h, respectively. At 96 h after inoculation the discs were treated with the antagonists (10^7 spores/ml), fungicide, and water plus surfactant, transferred to fresh PCA, and numbers of conidiophores were estimated after 7 days.

Hyphal interactions.

Possible interactions between B. cinerea and G. roseum, and between B. cinerea and T. viride, were examined by an agar plug technique and in leaf discs. Seven-mm plugs from colonies of the pathogen and antagonist on potato dextrose agar were positioned 3-cm apart on separation membrane (M.W. cut-off = 10 kDa) which overlaid a simple agar medium. The paired cultures were examined at intervals by light and phase-contrast microscopy. The leaf discs were inoculated with B. cinerea and three days later with the antagonists, then kept on PCA for 3 days. Discs were cleared, stained, and examined directly or after thin sectioning by light microscopy.

Biocontrol by mutants of G. roseum.

A parent isolate and two mutants (139 and 162) induced by ultra violet light were compared for effectiveness against B. cinerea in the leaf disc assay. The parent isolate produces an antifungal metabolite (low molecular weight, heat tolerant,

fungistatic) that suppresses B. cinerea; mutants 139 and 162, however, produce zero and relatively high levels of the metabolite, respectively (Peng 1991). G. roseum (parent and mutants) (10^6 spores/ml) were applied to discs 24 h before a challenge inoculation of B. cinerea (10^5 spores/ml).

Results

Biocontrol tests.

In the greenhouse, M. verrucaria, T. viride, and Penicillium sp. suppressed sporulation potential of B. cinerea in attached leaves by 97-100%, and as effectively as chlorothalonil (Table 1). Fusarium sp. and R. glutinis were moderately effective and ineffective, respectively.

Table 1. Effects of fungal antagonists and of chlorothalonil on sporulation potential of B. cinerea in strawberry leaves in the greenhouse and in field plots in 1989.

Treatments	Number of Conidiophores/ leaf disc (greenhouse)	Incidence of conidiophores in leaf discs for the field (%)	
		Overwintered leaves	Spring leaves
Water plus surfactant	120 a†	25.7 a	18.7 a
Rodotorula glutinis	112 a	27.0 a	16.4 a
Fusarium	28 b	23.0 a	23.6 a
Myrothecium verrucaria	2 c	22.0 a	14.0 a
Trichoderma viride	4 c	13.3 b	2.8 b
Penicillium sp.	3 c	9.3 b	0.0 b
Gliocladium roseum	0 c	10.7 b	0.0 b
Chlorothalonil	3 c	9.0 b	3.5 b

†$P \leq 0.05$, protected LSD.

In the field in 1989, T. viride, Penicillium sp., G. roseum, and chlorothalonil variously suppressed sporulation incidence of B. cinerea by 48-64% in overwintered leaves, and by 85-100% in leaves produced in spring (Table 1). Other fungi were ineffective. At the time of treatment the overwintered leaves were semisenescent and the spring leaves were green.

In field plots in 1990, T. viride, Penicillium sp., G. roseum and chlorothalonil each suppressed sporulation potential of B. cinerea in strawberry leaves (Table 2). Only G. roseum was as effective as chlorothalonil.

Temperature effects.

G. roseum, T. viride, and Penicillium sp. suppressed conidiophore production of B. cinerea with increased effectiveness as temperature increased from 10-25 C. Only G. roseum was markedly effective at 10 and 15 C (78 and 90% suppression, respectively). At 20 and 25 C each of the antagonists suppressed B. cinerea in the range of 92-100%, and about as effectively as chlorothalonil, which was highly suppressive at all temperatures.

Table 2. Effects of antagonistic fungi and of chlorothalonil on estimated sporulation potential of B. cinerea in strawberry leaves in the field in September 1990.

Treatment	Incidence of quarter-leaflets with conidiophores of B. cinerea: Leaves treated individually	Incidence of quarter-leaflets with conidiophores of B. cinerea: Inundative Applications
Water plus surfactant	49 a†	64 a
Trichoderma viride	20 b	30 b
Penicillium sp.	20 b	22 bc
Gliocladium roseum	0 c	7 d
Chlorothalonil	5 c	11 cd

†$P \leq 0.05$, protected LSD.

Rate of germination of each antagonist on leaf discs generally increased with temperature in the range tested and was most rapid at 25 C. Gliocladium roseum germinated faster than T. viride and Penicillium sp., especially at 10-20 C. For example, time to 90% germination at 15 C was 46, 109, and 99 h for the respective antagonists. Germ tubes also were markedly longer in G. roseum than in T. viride and Penicillium sp. after various incubation times at the various test temperatures.

Effects of leaf senescence and death.
The ability of the antagonists to suppress conidiophore production of B. cinerea was high (> 90%) and not affected when leaf discs infected with the pathogen were incubated on PCA for 24 h before the antagonists were applied. It was markedly reduced or eliminated however in discs incubated for 48 or 72 h. Chlorothalonil suppressed conidiophores almost completely in all PCA treatments.

Gliocladium roseum suppressed conidiophore production by B. cinerea by 88-100%, and as effectively as chlorothalonil, when applied to green or senescent leaves inoculated 48 h earlier with the pathogen, but failed to suppress conidiophores in similarly-treated dead leaves.

Hyphal interactions.
In the separation-membrane assay, Penicillium sp. inhibited B. cinerea without hyphal contact. No morphological changes were observed in hyphae of B. cinerea in presence of G. roseum and Penicillium sp. Hyphae of T. viride occasionally coiled around those of B. cinerea without forming penetration structures or penetrating the pathogen.

Hyphae of the three antagonists grew profusely on surfaces of leaf discs incubated on PCA and often penetrated the epidermis and colonized the mesophyll. Growth of B. cinerea was sparse in epidermal cells that were also invaded by antagonists, but often abundant in uninvaded cells. Relatively long hyphae (> 100 µm) of B. cinerea were infrequent in leaf discs treated with antagonists but frequent in water checks. No evidence of hyperparasitism was found.

Biocontrol by mutants of G. roseum.
The parent isolate, mutant 139 and mutant 162 suppressed sporulation of B. cinerea in leaf discs by 100, 98, and 90%, respectively, and did not differ significantly in effectiveness.

Discussion

The isolates of G. roseum, T. viride, and Penicillium sp. markedly suppressed sporulation potential of B. cinerea in strawberry leaves under a wide range of conditions in the greenhouse and in the field. The antagonists were effective even though they were applied 2-5 weeks after the leaves were inoculated with the pathogen. Only G. roseum, however, was consistently as effective as chlorothalonil which, in earlier studies, suppressed the pathogen in strawberry leaves better than other commercially available fungicides (Sutton 1990). The more consistent effectiveness of G. roseum was likely related to its ability to markedly suppress B. cinerea at 10 and 15 C as well as at 20 and 25 C.

The antagonists suppressed B. cinerea in infected leaves chiefly when applied while the leaves were green or senescent. All were ineffective when applied to leaves killed by incubation on PCA for > 48 h, and G. roseum failed to suppress the pathogen when tested on leaves that had died naturally. Biocontrol also was less effective in overwintered leaves than in young green leaves in field tests in 1989. Effectiveness of biocontrol generally declined sharply shortly before or after leaves died.

Collectively, the observations supported the concept that the antagonists suppressed B. cinerea when the pathogen invaded senescent or dead leaf tissues from sites of quiescent infection in the epidermis (Sutton 1990). When infected leaves were inoculated with antagonists and killed on PCA, the antagonists invaded the tissues, reduced growth of the pathogen in the tissues and suppressed sporulation potential of the pathogen. From the markedly reduced effectiveness of biocontrol when the antagonists were applied to infected leaves ≥ 48 h after the leaves were killed on PCA, when applied to leaves that had died naturally, and when applied to senescent overwinter leaves, the antagonists may suppress B. cinerea only while the pathogen is colonizing the tissues. Thus, the antagonists are likely ineffective in tissues already colonized or possessed by the pathogen.

Competition between the antagonists and B. cinerea for leaf substrate was probably a key mechanism of biocontrol. From frequent occurrence (McLean and Sutton 1992), and rapid growth of the antagonists in strawberry foliage, the fungi may be ecologically well-adapted and have high exploitive potential in the leaf tissues. While observations in the separation-membrane assay indicated that the isolates of G. roseum and Penicillium sp. suppress growth of B. cinerea through antibiosis, the observations of biocontrol of B. cinerea in leaf discs by mutants of G. roseum with enhanced or zero production of an antifungal metabolite indicated that antibiosis may not be important for biocontrol by this antagonist. No evidence was found to support possible hyperparasitism of B. cinerea. The observations of suppressed hyphal developed and sporulation of the pathogen in leaves treated with G. roseum, T. viride, and Penicillium sp. were compatible with concepts of exploitive competition and interfering competition.

Applications of G. roseum, T. viride and Penicillium sp. to strawberry leaves may be useful for managing gray mold fruit rot in the field. The antagonists suppressed sporulation potential of B. cinerea in the field as effectively or almost as effectively as chlorothalonil, which when applied to leaves in early spring suppressed fruit rot as effectively as conventional sprays applied when the strawberries are flowering and fruiting (Sutton 1990). From the effectiveness of

the antagonists in green or senescent leaves as opposed to dead leaves, it may be prudent to time applications to precede death of leaves of successive flushes (Sutton 1990).

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BIOLOGICAL CONTROL OF *BOTRYTIS* LEAF BLIGHT OF ONION

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Summary

Saprophytic antagonists can be applied to suppress the sporulation of *Botrytis* on necrotic tissue. The effect of sporulation suppression on an epidemic of *Botrytis* was studied in a field experiment with onions. When 30-50% of the necrotic tissue was removed from the field, the content of *Botrytis* conidia in the air above the crop was significantly reduced down to 34% compared to the control. The epidemic of *Botrytis* was significantly delayed. In late August, the number of lesions on the leaves was 1.1 lesions cm^{-2} in the control and 0.6 lesions cm^{-2} after removal of the necrotic tissue. In an additional treatment, a conidial suspension of the antagonist *Gliocladium roseum* was sprayed weekly, but this had no effect on the *Botrytis* epidemic.

A bioassay with dead onion leaves, preinoculated with *Botrytis aclada*, was developed to select antagonists amongst saprophytes isolated from necrotic onion leaves. Isolates from *Alternaria* spp., *Arthrinium* spp., *Chaetomium globosum* and *Ulocladium* spp. suppressed sporulation of *B. aclada* almost completely, whereas *Cladosporium* spp. and *Penicillium* spp. were less efficient. Also most isolates of *Gliocladium* spp. and *Trichoderma* spp., not isolated from necrotic leaf tissue, were highly efficient.

Introduction

Botrytis spp., sporulate primarily on dead tissue of the host plants. The necrotic tissue may also provide a suitable substrate for saprophytic antagonists. In this substrate, antagonists may interfere with the saprophytically growing mycelium of *Botrytis* and suppress sporulation of the pathogen. An effect of introduced antagonists on the epidemic of *Botrytis* can only be expected when most of the infections during the epidemic are caused by spores produced within the crop and inoculum produced outside the crop is less important.

In this paper, we present results from a screening of antagonists and from a field experiment with onions carried out to evaluate the reliability of the strategy of biological control of *Botrytis* spp. via suppression of sporulation.

Material and Methods

Field experiment. Onions were sown in plots of 9 x 12 m with 6 replicates per treatment. To minimize interplot interference, plots were separated by 12 m wide strips with sugar beets. The effect of antagonists was simulated by the artificial removal of necrotic leaf tissue in weekly intervals. Approximately 30-50 % of the substrate, suitable for sporulation of *Botrytis* spp., was removed during the growing season. In an additional treatment, *Gliocladium roseum* was applied with 1×10^6 conidia ml^{-1} in weekly intervals.

The development of the epidemics of *Botrytis* in the separated plots was followed by counting leaf spots of 10 plants per plot, sampled in weekly intervals.

The spore load in the plots was quantified during August when abundant sporulation occurred in the crop. Therefore, Rotorods (Edmonts, 1972) were used simultaneously in two plots per treatment in a height of 0.3 m. Two to four runs were carried out on five days between 10:00 a.m. and 1:00 p.m. when spore release could be expected (Lacy & Pontius, 1983). The concentrations of airborne spores were calculated from runs of 15 min.

Bioassay. Saprophytes isolated from necrotic leaf tips of onions (Mathar, unpublished) and strains of *Trichoderma* spp., *Gliocladium* spp. or yeasts were tested in a bioassay in order to select antagonists which are able to suppress sporulation of *Botrytis* spp. necrotic leaf tissue. Therefore, green onion leaves were dried, gamma-radiated and washed thoroughly to remove soluble nutrients. Leaves were inoculated with conidial suspensions of *B. aclada* (1×10^5 conidia ml^{-1}) and incubated for 24h at 18°C in moist chambers. These leaves precolonized by *B. aclada*, were sprayed with conidial suspensions of the antagonists (1×10^6 spores ml^{-1}). The percentage leaf area covered with conidiophores of *B. aclada* was estimated after an incubation period of another 7 days in moist chambers.

Results

Field experiment. After the epidemic of *Botrytis* started in the middle of July, the number of lesions increased exponentially until end of August (Fig. 1.). In plots were necrotic leaf tissue had been removed, the epidemic was delayed significantly with $p < 0.001$ for the differences between the slopes of the regression lines. In late August, 1.1. lesions per cm^2 were counted on leaves from the control plots, whereas 0.6 lesions were found on leaves from plots where necrotic leaf tissue had been removed. The application of *G. roseum* did not affect the epidemic of *Botrytis*.

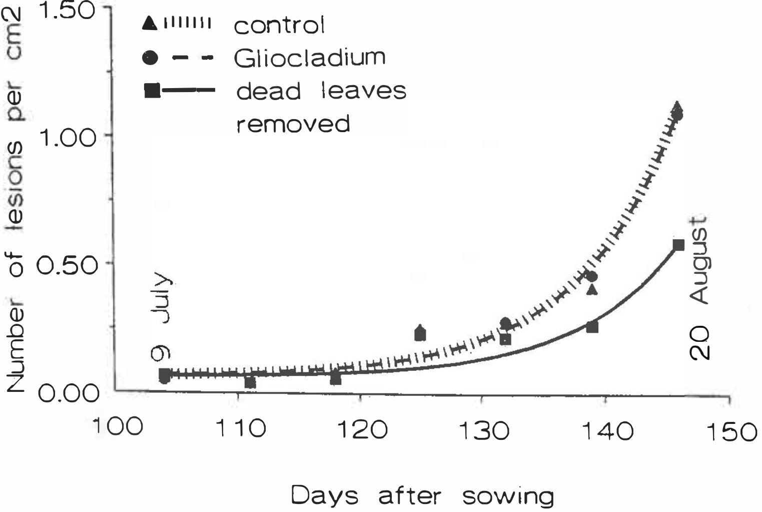

Fig. 1. Epidemics of *Botrytis* in onion plots after removal of necrotic tissue or applications with *Gliocladium roseum*.

The spore load in the air in plots where necrotic tissue had been removed was up to 66 % lower as compared to the control plots. A significant reduction of the spore load was mainly found during the peaks of the spore release (Fig. 2). The application of *G. roseum* did not result in a significant reduction of the spore load compared to the control.
Conidia trapped with Rotorods, washed from necrotic leaf tips or isolated from lesions mostly had a length of less than 15 μm. Thus, it can be assumed, that *Botrytis cinerea* was predominant in the field and the occurrence of *B. squamosa* was less pronounced (Ellis, 1971).

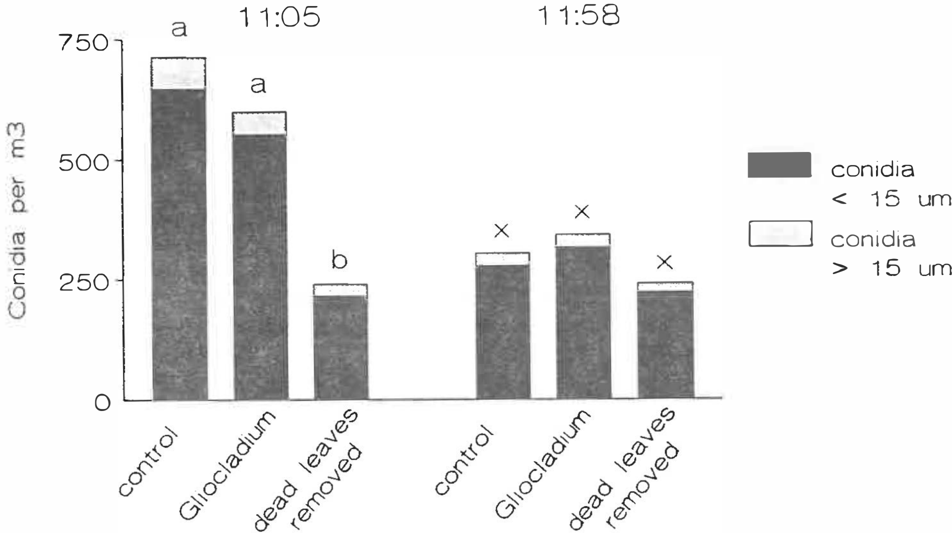

Fig. 2. Concentration of airborne conidia of *Botrytis cinerea* (< 15µm) and *B. squamosa* (> 15µm) after removal of necrotic leaf tissue or applications of *Gliocladium roseum*. Data of two runs at August 16. Columns with the same letter do not differ significantly according to LSD-test (p = 0.05).

Bioassay. In total 58 isolates belonging to 26 species were tested in bioassays. Strains belonging to *Gliocladium catenulatum*, *G. roseum*, *G. nigrovirens*, *Trichoderma viride*, *Alternaria alternata*, *A. infectoria*, *Chaetomium globosum*, *Ulocladium chartarum* and *U. atrum* suppressed the sporulation of *B. aclada* almost completely. Isolates of *Cladosporium cladosporioides*, *Penicillium* spp., some isolates of *Ulocladium chartarum*, some isolates of *Aureobasidium pullulans* and yeasts were weak antagonists, whereas *Arthrinium montagnei*, *A. phaeospermum*, *Cladosporium herbarum*, *Sesquicillium candelabrum* and *Trichoderma harzianum* showed intermediate results. The effect of antagonistic saprophytes on the sporulation of *B. aclada* in one of the experiments is shown as an example in Tab. 1.

Discussion

A clear relationship between the amount of suitable substrate for sporulation of *Botrytis* spp., the *Botrytis* spore load in the air above the crop and the number of lesions on leaves could be found in the field experiment. Thus, an epidemic of *Botrytis* spp. in onions mainly depends on conidia produced inside the field on the host plants. This seems not only true for the host specific *B. squamosa* but also for the non-specific, ubiquitous *B. cinerea*, which was dominant in our field experiment.
Necrotic tissue is a much more attractive substrate for saprophytic antagonists than the surface of an intact leaf (Fokkema *et al.*, 1992). Consequently, biological control of *Botrytis* diseases by suppression of sporulation seems to be more feasible than the use of antagonists to prevent infections on the green leaf. Along this line, Peng & Sutton (1990) found that spraying with *Gliocladium roseum* was equally effective as fungicides in reducing sporulation of *B. cinerea* on dead leaves and fruit rot incidence in strawberry fields. *G. roseum* was not effective under our experimental conditions. This can be due to the spraying technique, to the quality of the conidia

applied or to the ecological competence of the antagonist, which is primarily a soil habitant.
Promising antagonists could be selected during the screening amongst saprophytes naturally occurring on necrotic leaf tissue as well as amongst *Gliocladium* spp. and *Trichoderma* spp.
In further experiments, the ecological competence of the antagonists and their performance under field conditions will be tested.

Table 1. Suppression of sporulation of *Botrytis aclada* by antagonists in a bioassay based on dead onion leaf segments precolonized by *B. aclada*.

Treatment	Percentage leaf area covered with conidiophores of *B. aclada*
control	85
Alternaria alternata 300	1
A. alternata 305	4
A. alternata 310	1
Arthrinium montagnei 242	11
Aureobasidium pullulans 494	63
Chaetomium globosum 256	0
Cladosporium cladosporioides 549	48
C. cladosporioides 564	40
Gliocladium catenulatum 162	0
G. roseum 016	0
G. roseum 160	0
G. roseum 161	0
Ulocladium atrum 385	6
U. chartarum 355	18
U. chartarum 380	26
$LSD_{5\%}$	15

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SECONDARY MICROBES IN LEAF LESIONS OF *DRECHSLERA TERES.*

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Summary

A study of the behaviour of secondary colonisers of *Drechslera teres* leaf lesions on barley was carried out in June and July. It was found that bacteria and yeast population behaviour in small lesions was not obviously related to behaviour in large lesions. Total numbers per unit area of lesion varied apparently independently in the two lesion categories as did total numbers per lesion. In the first year sample the secondary organisms from the July sampling were more suppressive of lesion extension and sporulation when re-introduced into lesions caused by *D. teres.* These organisms showed a different enzyme repertoire profile than the profile found at the June sampling. Some ideas relating to these observations are discussed.

Introduction

It was thought possible that microbial saprophytes in leaf lesions might react in a similar fashion accredited to macro-organisms in the theory of Island Ecology (MacArthur & Wilson 1967) and further that leaf lesions might possibly provide a more stable habitat than leaf surfaces. There are a lot of references in the literature on leaf surface microorganisms but a dearth of information on leaf lesion micro organisms or at least on their populations. The possibility that older leaf lesions might support organisms which were more highly competitive than those supported on young leaf lesions existed and further that some of these organisms might affect the activity of the pathogen causing the lesion.

Materials and Methods

Winter barley crops were sampled in June and July of two consecutive years. Small lesions (SL) of *Drechslera teres* were classified as those less than 10mm diameter, large lesions (LL) as those of greater than 10mm. Fifty SLs and fifty LLs were selected from sites throughout the crop at each sampling date. Lesions were cut from the healthy leaf. 1 ml and 4 mls of Ringers soln was dispensed respectively onto small and large lesions. The lesions were then washed on a Whirlimix for 15 secs and the washings were subjected to a dilution series. 0.1 mls was then plated onto NA giving total colony forming units (CFUs) and on NA containing antibiotics giving yeast CFUs. From the total CFUs a random sample was selected and these were inoculated (1×10^6/ml) into artificially produced lesions of *Drechslera teres* (5x5 latin square design to rule out any problems such as varying susceptibility of leaf area). Sporulation and lesion extension was then recorded in these lesions. The isolated micro-organisms were further subjected to a series of enzyme tests with a view to identifying their "enzytype". Enzymes tested for were cellulase (A), (Zucker & Hankin 1970), pectate lyase (D), polygalacturonase (E), (Hankin et al 1971), amylase (C), chitinase (B), lipolase (F), ligninase (G), proteolytic activity (H) and urease (I), (Cowan & Steele 1974).

Results and Discussion

Table 1. Overall population trends of microbial saprophytes in *Drechslera teres* leaf lesions in June and July.

	Total CFU's x10^5	Total CFU's/mm^2	Yeast CFU's
June Yr 1	SL > LL		LL > SL
July Yr 1	LL > SL		N.S.
June Yr 2	LL > SL	LL > SL	SL > LL
July Yr 2	LL > SL	SL > LL	SL > LL

Table 2. Number of isolates (/150 tested) originating from small or large *Drechslera teres* leaf lesions in June or July year 1 which reduce lesion extension in artificially produced lesions.

YEAR 1			
JUNE		JULY	
Small Lesions	Large Lesions	Small Lesions	Large Lesions
62	61	111*	105*

* $P < 0.005$, level of significance for the difference with June treatment.

Table 3. Number of isolates (/150 tested) originating from small or large *Drechslera teres* leaf lesions in June or July year 1 which reduce sporulation in artificially produced lesions.

YEAR 1			
JUNE		JULY	
Small Lesions	Large Lesions	Small Lesions	Large Lesions
83	78	148*	150*

* $P < 0.005$, level of significance for the difference with June treatment.

In the SLs the lesions are just becoming necrotic, the dying boundary makes up all of the lesion whereas in the LLs this only makes up a small portion of the entire area. The question was posed as to whether LLs and SLs supported different microbial populations and from Table 1 we can see that these populations do appear to operate differently and if there is some governing "rule of thumb" which determines the make up of the population, then there are two different rules as far as LLs and SLs are concerned. Although it is obvious that there are differences and trends one cannot predict in which direction the trend will go. Note the yeast numbers with respect to the total CFUs, there is an inverse relationship between these two columns in June yr1, June yr2 and July yr2 and where this isn't the case - July yr1, there is no significant difference between the yeasts supported on SLs and LLs. In the two consecutive sampling years yeasts rather consistently made up a higher proportion of the total population where the total population was low. In testing the hypothesis that LLs may support a microflora whose make up would be such that it contained a higher proportion of the more competitive type of organism, there was no significant difference in the action of LL and SL isolates within the same

sample date (Tables 2 & 3). However SL and LL isolates obtained in July were significantly more suppressive than isolates obtained in June.

In the literature the view is being taken that organisms first colonising islands are r-selected or opportunistic species, while the later ones are seen as being K-selected and of a more competitive nature. In implementing this theory we must remember that the pathogen and isolate may or may not be on the same trophic level but the suppression may be as a result of generalised competition by the isolates, maybe antibiosis occurring and possibly as with the two elephants fighting - the grass gets trampled! Perhaps there is a point in lesion development when nutrients are in scarce supply and for the saprophyte it becomes profitable to act against the pathogen and utilise its resources or stop its sporulation.

The difference in the suppressiveness of these isolates might be reflected in a difference in the species make up. When the enzyme tests for each isolate were combined it was possible to gain an insight into their enzyme repertoire and this enabled us to establish what at least may be termed "enzytypes". It should be noted that bacteria undergo various phases and that enzytype may not be constant within the same microbe. Each phase may not produce the same enzymes but there still remains the possibility of ecological equivalence of isolates. In the June sample there were favoured enzytypes, in order of abundance: CFH, CH, FH, H, CFHI and CHI. In the July sample some of these enzytypes persisted; CFH, CFHI, CH but mostly at lower frequencies. There were some extinctions between the two samples and also some new colonisers. One very interesting new introduction was a class which showed negative to all of the above enzymes - termed "blank" and represented the most abundant enzytypes. Because of the fact that our enzyme tests are on solid media and as a positive result is recorded by a zone around the colony, we may say that these are organisms producing extracellular enzymes. If it were cellular or cytoplasmic enzymes being produced, these would not show up on our tests. Thus this data may be interpreted as a progression to organisms with cellular enzymes. It is possible to say that later on in the season when nutrients are low in the lesion, it is a wasted expense to ooze enzymes. Localised enzyme application may be seen as a much more conservative and efficient lifestyle for organisms living on a depleting food source.

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ASPECTS OF SELECTION AND USE OF FUNGAL ANTAGONISTS FOR BIOLOGICAL CONTROL OF *SCLEROTINIA SCLEROTIORUM* IN GLASSHOUSE CELERY AND LETTUCE

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Summary

At HRI Littlehampton, fungal antagonists of *Sclerotinia sclerotiorum* are isolated either by baiting soil with sclerotia of *S. sclerotiorum* or by placing soil crumbs onto potato dextrose agar (PDA) plates colonized with *S. sclerotiorum.* Antagonism is subsequently assessed by measuring mycelial growth of antagonists across *S. sclerotiorum-precolonized* PDA plates and by quantifying their ability to infect sclerotia of *S. sclerotiorum.* A bioassay to assess the ability of antagonists to prevent growth and subsequent sclerotia formation of *S. sclerotiorum* in plant tissue pieces may also be used to provide information about the ecology of the antagonist and the likelihood of the antagonist to give biocontrol of *S. sclerotiorum* on plant debris. Results involving the use of *Coniothyrium minitans* and *Trichoderma* spp. applied before planting to soil as maizemeal-perlite inocula for the control of *S. sclerotiorum* in glasshouse celery and lettuce crops are presented. Possible future research in this area is discussed.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a pathogen of over 360 plant species worldwide (Purdy, 1979) and under suitable environmental conditions can cause catastrophic crop losses. The pathogen survives between crops or overwinters as sclerotia in the soil or within plant debris. The sclerotia may germinate myceliogenically to infect roots, crowns and stem bases or senescent leaves as they touch the ground. Alternatively, sclerotia may germinate carpogenically, producing apothecia from which ascospores are released. These ascospores then germinate on petals, senescent leaves or damaged parts of the plant where nutrients are being released, leading to aerial infections. This latter mode of germination and infection is important within celery and lettuce crops in the UK. The disease then spreads rapidly by plant to plant contact.

Control is normally achieved by repeated fungicide sprays which prevent ascospore infections. If the disease builds up, the soil may be fumigated or steamed to eliminate sclerotia. However, the number of effective fungicides available is gradually decreasing, soil fumigation with methyl bromide is becoming environmentally unacceptable and steaming is still extremely expensive. Consequently, the development of alternative biocontrol strategies is becoming increasingly important. These can be targeted at the site of initial ascospore infection on the plant (Zhou & Reeleder, 1989) or at the control of sclerotial populations in the soil or plant debris. This latter strategy has been the one of choice at HRI-Littlehampton.

This paper briefly describes some of the strategies used routinely at HRI-Littlehampton for the selection and assessment of efficacy of fungal biocontrol agents of *S. sclerotiorum* for use in glasshouse crops. Areas for future research are also considered.

Isolation of antagonists

Antagonists were isolated from soil either by baiting with sclerotia of S. sclerotiorum using the method of Whipps et al. (1993) or by use of S. sclerotiorum-precolonized PDA plates based on the procedures of Deacon & Henry (1978) and van den Boogert & Gams (1988). In the first case, sclerotia were placed in moist sieved soil in Petri dishes and incubated for 4 weeks at 18°C. When they occurred, fruiting structures of mycoparasites on the surface of sclerotia were removed and cultured on acidified Czapek-Dox agar. Subsequently, all sclerotia were surface sterilized, bisected and placed onto agar plugs of PDA + aureomycin (Whipps & Budge, 1990) and mycoparasites again isolated and subcultured. In the second case, soil crumbs (2 mm^3) were placed on the periphery of a PDA plate just colonized by S. sclerotiorum. After 4 weeks incubation at 18°C, mycelial outgrowths from the crumbs were subcultured onto PDA + aureomycin.

Assessment of efficacy

Two methods were used to evaluate efficacy of putative antagonists based on the ability to infect sclerotia (Whipps & Budge, 1990) and growth across a S. sclerotiorum-precolonized PDA plate (Deacon & Henry, 1978). All experiments were done at 18°C. Firstly, spore suspensions of the antagonists grown on PDA were prepared in sterile distilled water ($\simeq 10^6$ spores ml^{-1}). Sclerotia of S. sclerotiorum were incubated in the spore suspensions for 30 min and were then placed in moist sterile sand in a Petri dish and incubated for 3-4 weeks. The sclerotia were then surface sterilized, bisected, placed onto PDA + aureomycin and assessed for viability and presence of the antagonist. Secondly, Petri dishes containing PDA were inoculated at the margin with S. sclerotiorum and when the colony had just reached the opposite edge of the dish, a 5 mm diam PDA disc of the antagonist was placed on the young colony margin of the pathogen. Growth across the colony of S. sclerotiorum was then assessed after 7, 14 and 21 days by removing a strip 5 mm wide in a line from the inoculum disc of the antagonist to the inoculum position of S. sclerotiorum each week. The strip was then cut into 5 mm segments and assessed for the presence of the antagonist, thus enabling growth rates of the antagonists to be calculated. From these two techniques a ranking system based on (i) the ability to infect sclerotia and (ii) growth across precolonized plates, both attributes considered useful characteristics for biocontrol agents of S. sclerotiorum, could be constructed. Isolates with high activity in either one or other, both or neither of the tests were detected.

Tissue piece bioassay

As S. sclerotiorum produces sclerotia on senescent plant tissue, an additional approach to control in the soil could be to apply either a biocontrol agent that could occupy senescent tissue before S. sclerotiorum, thus preventing colonization and growth of S. sclerotiorum, or to apply one that will grow in S. sclerotiorum occupied tissue, killing or inhibiting growth of the pathogen and thus preventing sclerotia formation or decreasing sclerotia viability. A tissue piece bioassay was devised to assess antagonists quantitatively for these characteristics (Whipps, 1987). Briefly, a PDA disc of antagonist was placed on a PDA disc of S. sclerotiorum at the same time, one day before or one day after so that both discs were in contact with the cut surfaces of three segments (2 cm long) of either tomato stem, celery stem or lettuce petioles. Growth of the pathogen was assessed microscopically and, after 4 weeks, presence of pathogen and antagonist within the distal end of the segment was assessed by plating samples onto PDA + aureomycin.

The number of sclerotia produced was counted and sclerotial viability and infection by antagonists assessed as described earlier. Antagonists with ability to occupy plant tissue, prevent sclerotia formation and infect sclerotia were found. This emphasises the possibility of developing screens for biocontrol agents based on ecological grounds where the etiology of the pathogen and cropping system in use are considered.

Glasshouse trials

Six years of glasshouse trials involving preplanting applications of maizemeal-perlite preparations of Coniothyrium minitans and Trichoderma spp. for the control of S. sclerotiorum in celery and lettuce have been reviewed recently (Whipps, 1992). A few key conclusions can be drawn from this work. C. minitans gave reproducible control of S. sclerotiorum in lettuce equivalent to repeated spray treatments with the fungicide vinclozolin when disease levels were low but it failed to match the fungicide treatment at high disease levels, even though significant reductions in disease were obtained. Even so, C. minitans survived and spread in the glasshouse, infected sclerotia of S. sclerotiorum at all times of the year, decreasing survival when the soil was moist and reducing sclerotial viability and apothecial production. The long duration (3-4 months) of cropping of celery may prevent effective disease control by C. minitans in this crop. In contrast, none of the Trichoderma spp. gave any control even though they survived in the soil. This may be related to the relatively low temperatures in the glasshouse soils (*circa* 18°C at 2 cm depth) in comparison with 28°C used in original sclerotia infection tests in France (Davet, 1986). Indeed, Trichoderma strains HH3 and Bl used in these glasshouse trials also performed poorly in comparison with C. minitans and Gliocladium virens G20 in sclerotia infection tests carried out at Littlehampton at 18°C (Whipps & Budge, 1990). This again emphasises the need to set up screening systems which realistically match the environmental conditions where the biocontrol agent is required to act.

Conclusions and future work

At HRI at the moment, a range of fungal isolates with biocontrol potential against S. sclerotiorum are at different stages of assessment and development. C. minitans must be viewed as a lead strain as it has reproducible activity in glasshouse trials. It has also performed well in the sclerotial infection tests and tissue piece bioassays providing some credence to the screening procedure. However, it also demonstrates that several selective screens, based on different biocontrol attributes need to be carried out when searching for a disease biocontrol agent, for if C. minitans had been tested solely in the precolonized plate test, it would have rated very poorly. Nevertheless, a major problem with this fungus at the moment is that it has to be grown and applied on a solid substrate. Although, it can be cultured in liquid fermentation, it sporulates poorly and it is likely that the spores are the key propagules for its biocontrol activity. In contrast, G. virens G20, which performed well in sclerotial infection tests, is easy to grow in liquid culture, can be readily formulated and a great deal is known about its physiology (Jackson et al., 1991). In addition, it has biocontrol activity against other pathogens and has now been released commercially in the USA for control of damping-off in bedding plants. This background knowledge coupled with ready application must encourage its use in further experiments for the control of S. sclerotiorum.

A range of strategies may be applied in the future to obtain commercially viable biocontrol agents of S. sclerotiorum. Having produced a screening system that seems to work, more antagonists with the same attributes and greater activity could be selected. In addition, the physiological and environmental parameters for optimum growth, inoculum production and survival should be investigated. Perhaps finally, useful isolates could be selected or even genetically manipulated or mutagenized to provide fungicide resistant strains that could be integrated with fungicides currently in use in the glasshouse. Such approaches are being investigated at the moment.

Acknowledgements

This work was supported by the Ministry of Agriculture, Fisheries and Food.

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MICROBIAL SUPPRESSION OF VIABLE SCLEROTIA OF SCLEROTINIA SCLEROTIORUM AND WHITE MOULD DISEASE IN FIELD CROPS.

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Summary

The effect of application of the antagonist Coniothyrium minitans to Sclerotinia sclerotiorum infected crops was studied.

In a 5-year experiment on S. sclerotiorum infested soil in the North-East Polder, potato, bean, carrot and chicory were grown in rotation in this order. Spraying of C. minitans on S. sclerotiorum infected crops led to infection of newly formed sclerotia. This could be demonstrated by scoring the presence of C. minitans in such sclerotia after plating on agar media, and by counting apothecia - the visible sign of soil infestation by sclerotia - in the field. With both methods viability of sclerotia proved to be reduced to less than 10% of the control. With direct laboratory scores it made no difference whether sclerotia were collected from within plant structures, such as stems or pods, or on the plant. The apothecia count showed the effect of application of the antagonist with a delay of two years, due to turning the sclerotia to deeper soil levels and back by tillage operations. Trichoderma was ineffective in reducing the viability of sclerotia.

One single spray of C. minitans in autumn of the first year of a biennial caraway crop resulted in a reduction of the number of apothecia in spring of the following year from 7.2 to 2.0 per 18 m row length. Repeated C. minitans sprays in spring of the second year gave a plant density dependent disease reduction from about 12% disease incidence at low plant density and of the control to 1% in the most dense crop.

It is concluded that C. minitans is a promising biocontrol agent. It will be useful in a long term perspective by preventing soil contamination after a S. sclerotiorum infected crop, and in special cases it might even directly reduce disease in the treated crop.

Introduction

Sclerotinia sclerotiorum, the causal organism of white mould, survives as sclerotia in soil. It has a very broad host range, which precludes crop rotation as an efficient means of dealing with soil infestation, except for rotations with a high cereal share. Even low numbers of sclerotia in the surface layers of the soil can cause widespread disease through apothecia-borne ascospores which are spread by air currents. Prevention of soil infestation, but only if rigorous, therefore is a valuable approach to manage the disease. Research at IPO-DLO primarily aims at preventing soil infestation by treatment of white mould diseased crops with antagonists of S. sclerotiorum. The mycoparasite Coniothyrium minitans is the choice biological control organism (Whipps & Gerlagh, 1992).

Materials and methods

Model crops for the field trials were bean (Phaseolus vulgaris) and caraway (Carum carvi). The bean crop was included in a 5-year field experiment with four crops, potato, bean, carrot and chicory, in rotation in this order. Especially bean is very susceptible and assures a high production of sclerotia. Caraway is a biennial crop, which causes sclerotia produced on the vegetative phase or the cover crop in the first year to be deposited and to remain on the soil surface, thus contributing to infection by ascospores in the second year. Crops were sprayed three times at the end of their cycle, from flowering on, with spore suspensions $2\text{-}5.10^6$ conidia ml^{-1} of C. minitans, but the caraway crop was also sprayed at the end of the first year. The success of sprays was monitored by collecting fresh sclerotia, which were subsequently surface sterilized, bisected, plated on PDA, and scored for growth of S. sclerotiorum or the antagonist (Whipps & Budge, 1990). The number of viable sclerotia per surface area of field soil was scored by counting apothecia. Since the maximum amount of apothecia was reached at different times depending on crop characteristics (leaf canopy density) and weather conditions, data of the maximum counts in each crop, and not of the same date, were compared. Since normal tillage operations turn the soil, relevant counts are actually made two years after the spray with the antagonist, since most sclerotia will be at deep soil levels the first year.
In comparison to C. minitans sprays were also performed with supposedly antagonistic Trichoderma spp. The latter isolates, just like those of C. minitans, were obtained from sclerotia of S. sclerotiorum recovered from soil with a history of S. sclerotiorum infestation (Gerlagh & Vos, 1991).

Results and discussion

1. Viability of fresh sclerotia.

Sclerotia were collected from diseased bean plants at various periods after the antagonist sprays. The C. minitans but not the Trichoderma treatments resulted in a high percentage of the sclerotia infected by the antagonist. This percentage increased with later samples to more than 90% (Table 1). Low percentages of C. minitans in the other treatments originate from spontaneous contamination.

Sclerotia from inside stems and pods were only sampled at the last sampling date. The data show that these have been infected by C. minitans to the same degree as those on the plants. This is an important fact, especially with regard to some crops where most of the sclerotia are formed within the stems, such as sunflower and potato. The antagonist has the capacity to colonize tissue which has first been infected by S. sclerotiorum (Trutmann et al., 1982, Anon., 1992), and thus can penetrate the infected stem tissue to reach the sclerotia. The same observation has been documented by Huang (1977) and Merriman et al. (1979). Huang (1977) found less infection of sclerotia in the pith cavity of the basal stem by C. minitans but not in the pith cavity of taproots than on the root surface of sunflower. Merriman et al. (1979) found lower numbers of fungi on sclerotia formed within bean stems than on surfaces of bean plants. However, C. minitans, was the only exception among nine fungi specified.

Table 1. Effect of treatment of a S. sclerotiorum infected bean crop with antagonists on viability of sclerotia on and in the crop.

Dates of antagonist sprays: 15 July, 29 July, 12 August.

Treatment	Sampling date					
	12 August		26 August		8 September	
	S. scl.	C. min.	S. scl.	C. min.	S. scl.	C. min.
C. minitans on[1]	36[2]	64	46	54	9	91
C. minitans in[1]	-	-	-	-	7	93
Trichoderma spp.	100	0	88	12	89	11
Control	96	4	96	4	98	2

- not sampled
[1] on and in refer to sclerotia sampled on or in the diseased plant.
[2] figures are percentages of sclerotia viable (S. sclerotiorum) or infected by the antagonist C. minitans. Sample size between 25 and 50 sclerotia.

2. Effect of C. minitans sprays on emergence of apothecia.
Of the four susceptible crops in the rotation, potato, bean, carrot and chicory, only bean was heavily infected by S. sclerotiorum in each year. The very low numbers of apothecia in most crops (0.1 to 0.7 per 5 m row length), reflected the low disease severity two years earlier, and no effect of C. minitans sprays was found. However, in chicory, grown two years after bean, C. minitans significantly reduced the number of apothecia to 0.3, compared to 5.4 and 3.9 for Trichoderma treatment and control respectively (Table 2).

Table 2. Effect of antagonist sprays on S. sclerotiorum infected crops on numbers of germinated sclerotia in crops grown two years later.

Crop (actual)	Treatment		
	C. minitans	Trichoderma	Control
potato	0.3	0.7	0.4
bean	0.1	0.6	0.3
carrot	0.2	0.4	0.1
chicory	0.3	5.4	3.9

(Data represent counts per 5 m row length; means of 10 plots.)

The origin of the isolates of Trichoderma spp., sclerotia recovered from soil which had a history of S. sclerotiorum infestation, clearly is no guarantee for antagonistic capacity. Laboratory tests proved the lack of activity not to be caused by limited survival of the sprayed conidia in the field, for even with artificial infection of sclerotia in the laboratory, these isolates were ineffective (data not shown).

3. Effect of C. minitans on number of apothecia and disease incidence in biennial caraway.

In biennial caraway a single autumn spray with C. minitans reduced the number of apothecia in the following spring from 7.2 in the control to 2.0 per 18 m row length. Spring applications of C. minitans in the second year had an inverse plant density dependent effect on disease, superimposed on an average decrease in disease incidence from 11.9 to 5.1%. The latter reduction may partially be caused by the reduction in apothecia number. The density dependent decrease in disease incidence caused by C. minitans was from 12% at the lowest plant density (100-200 plants per 18 m row length) to 1% at the highest (>400-500 plants per 18 m)(Table 3). Most likely this reflects the better establishment of C. minitans in the denser, and therefore more humid crop. This is a rare case of a direct effect of C. minitans on disease under field conditions.

Table 3. Effect of C. minitans spray of a biennial S. sclerotiorum infected caraway crop in autumn of the first and spring of the second year on apothecia and disease incidence in the second year.

Plant density[1]	number of apothecia[1]		disease incidence[2]	
	C. minitans	Control	C. minitans	Control
100-200	2.3	2.3	12	7
>200-300	0.7	6.3	6	10
>300-400	6.0	10.7	4	17
>400-500	2.3	9.2	1	9
average	2.0	7.2	5.1	11.9
	r=0.329	r=0.330	r=-0.725	r=-0.055
	n.s., df=10	n.s., df=10	*, df=10	n.s., df=10

[1] numbers per 18 m row length
[2] in percentage
For each column the correlation has been calculated between plant density and the column variate. * = significant at α = 0.05; n.s. = not significant.

Conclusions

C. minitans establishes itself in S. sclerotiorum infected crops, and causes the decline of sclerotia on which S. sclerotiorum fully depends for its survival. In a dense caraway crop decreased survival of resting structures was accompanied by a strong effect of repeated C. minitans sprays on disease itself. These two phenomena prove the potential of C. minitans as an effective biocontrol agent.

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DISCUSSION: MICROBIAL SUPPRESSION OF DISSEMINATION OF NECROTROPHS

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The two papers on the reduction of sporulation of *Botrytis* spp. by saprophytic fungi convincingly demonstrated the potential of this strategy in the biological control of botrytis diseases. *Gliocladium roseum* appeared under field conditions the best antagonist in controlling sporulation in dead tissue of overwintering strawberry leaves. This may be different on other hosts. Foliar application of *G. roseum* to field-grown onions did not effect the *Botrytis* spore load above the crop nor the severity of leaf spot, whereas artifically removal of dead leaf tips did. *Gliocladium* spp. suppressed sporulation of *Botrytis* in bio-assays on dead onion leaves only under constant conditions of high humidity. This discrepancy between the performance of *G. roseum* on strawberry leaves and that on onion leaves can be explained by the apparent latent infection of still green strawberry leaves by *G. roseum*. This endophytic behaviour might be typical for strawberry leaves and may protect the antagonist from adverse micro-climatic conditions and may favour rapid colonization of dead tissue as soon as the leaf dies. In other hosts, like onion, other saprophytes which are more adapted to the specific conditions of dead leaf tissue should be used.

Biological control of *Sclerotinia sclerotiorum* by *Coniothyrium minitans* was very promising under glasshouse as well as under field conditions. Under low disease pressure *C. minitans* was equally effective as vinclozolin in controlling *Sclerotinia* rot in glasshouse-grown lettuce. In several field-grown crops, *C. minitans* colonized diseased plant tissue and inactivated the sclerotia produced in this tissue, resulting in a considerable reduction of soil-borne inoculum in the following seasons. A possible disadvantage for the development of a biocontrol product is the present inability to produce large quantities of conidia of *C. minitans* in liquid fermentation, which is no problem on solid substrates. In this respect it was argued that *Gliocladium virens* may have advantages, although its effect under practical conditions still has to be demonstrated. Further, it should be realized that *G. virens* can be an opportunistic pathogen of potatoes.

Strategies of biocontrol of foliar diseases

Control of biotrophs by mycoparasites

CONTROL OF BIOTROPHS BY MYCOPARASITES

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The number of publications on biological control increased steadily during the last years. This number, about 4555 publications during 1991, suggests that we are close to a major breakthrough in this area of research. In field experiments, however, it seems that biological control of biotrophic pathogens is still far from being successful. Numerous problems remain to be solved. Some examples are demonstrated here for rust fungi.

As a first step of host infection, rust uredospores produce an adhesion pad within 30 minutes after contact with the host cuticle (Deising et al., 1992). Thus, uredospores glue to the cuticle before germination starts. Only during the few hours of spore adhesion and subsequent growth of the germ tube on the plant leaf surface, microorganisms may influence fungal growth.Stimulation (Parker and Blakeman, 1984) or inhibition of spore germination and germ tube growth have been reported (Baker et al., 1983; Govindasamy and Balasubramanian, 1989; Kempf and Wolf, 1989; Levy et al., 1989; Rytter et al., 1989; Ghewande, 1990). Only few publications indicate the actual densities of antagonist and pathogen on the host leaves. In general, high numbers of antagonistic propagules have to be applied in order to be effective during the first hours of fungal growth on the leaf. The ratio spores of antagonist to spores of the pathogen on the leaves is given only rarely. If culture fluids of antagonists are applied, they have not been diluted and often, no actual concentration of the active ingredient is given. Thus, biological control is not easily manageable under practical conditions and a comparison with fungicide treatments is difficult.

Within a period of 12 hours, rust fungi produce appressoria and penetrate into the host plant. At this point, most antagonistic microorganisms become ineffective. As an alternative strategy, the induction of plant defence reactions with inducers has been attempted (Schönbeck and Dehne, 1986). However, this strategy does not provide complete control.

After penetration, the pathogen can be attacked again as soon as spores are produced in pustules erupting from below the epidermis. Metabolites of some fungi induce the precocious production of teliospores (Forrer, 1977). Once spores are produced, the pustules are hydrophobic and do not allow parasitic fungi to develop unless during very humid conditions. In spite of this, numerous fungi have been recorded to grow on such pustules (Saksiriat and Hoppe, 1990; Singh et al., 1990; Subrahmanyam et al., 1990; Saksiriat and Hoppe 1991; Stavely and Batra, 1991; De Nooij and Paul, 1992). Again, for the control of rusts, high concentrations of such fungi were applied. In spite of many optimistic claims, I do not believe that the application of such fungi is an efficient way to reduce rust incidence under natural conditions. The hyperparasitic fungus will always be lagging behind the development of the plant pathogenic fungus (Grabski and Mendgen, 1985). A reduction of disease around 50% is not sufficient.

To my opinion, biological control of rust diseases has the major disadvantage that antagonistic microorganisms can be efficient only during the germination of rust spores and much later during sporulation. There seems to be no effect during proliferation of the pathogen within the leaf. Also, quite variable conditions may prevail during these different stages of pathogen development. It is hard to imagine that any antagonist can compete with an effective fungicide under such conditions.

Powdery mildew fungi colonize their host plant in a different way. Exept for the haustoria, all fungal structures are superficial and can easily be reached by mycoparasites.The following talks will show us, whether there is a better chance for the biolobical control of these fungi.

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MYCOPARASITISM OF POWDERY AND DOWNY MILDEWS.

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Summary
Mycoparasitism of some fungi, especially *Tilletiopsis minor*, on cucumber powdery mildew is discussed. The importance of formulations is stressed. Some remarks on mycoparasitism of and induced resistance to downy mildew on red cabbage are made.

1.Powdery mildews.

Powdery mildews (Erysiphales) constitute a vast group of fungi, parasitizing many plant species all over the world. They cause great losses to plants in cultivation and require many tonnes of fungicides each year.
In Europe, powdery mildews are of great economic importance on cucumbers, other cucurbits, tomatoes, roses, chrysanthemums, grapes and cereals.

On cucumbers, powdery mildew (*Sphaerotheca fuliginea*) is a most serious pathogen. There are some resistant varieties, but these cannot be used in all seasons. Usually, susceptible varieties are grown, with the concomitant intensive use of fungicides. As the use of fungicides is increasingly criticized by consumers and environmental organisations, alternatives must be investigated. The method under development, in all probability, will have possibilities for adaptation to other crops.

Biological control of powdery mildews is possible by using mycoparasites (Burge, 1988; Philipp, 1988). Mycoparasites are fungi thriving upon other fungi, thus decreasing the growth and multiplication of the hosting fungi.

Mycoparasitism is a rather common phenomenon. Worldwide there are probably thousands of fungi parasitizing other fungi. Especially in autumn, when humidity increases, many can be found in the field.

Mycoparasites are usually not very specialised. Most of them will thrive on a broad range of powdery mildews and often on many other fungi as well. Some of them, like *Verticillium lecanii*, may infect insects and cysts of nematodes as well.

Temperature ranges of mycoparasites may vary considerably. Some, especially those collected from mushrooms in autumn, hardly grow above 25°C. Those isolated from powdery mildews in greenhouses usually have higher optima and maxima. One of the fungi we studied still grows at 37°C.

Fungal cell walls are assumed to consist mainly of cellulose, chitin and ß-(1,3)-glucan. As production of hydrolytic enzymes might be important, we studied some glycolytic activities in a number of mycoparasites (Hijwegen, 1992a). Cellulolytic enzymes don't seem to be very important in the parasitism of powdery mildews: the best cellulase-producers had the poorest performance on powdery mildews. Most of the parasites studied produced chitinases, but there was no correlation between chitinase activity and parasitic activity. However, we found a good correlation between parasitic activity on cucumber powdery mildew and ß-(1,3)-glucanase production assessed on laminarin as a substrate. The most effective mycoparasites dissolved laminarin most rapidly.

We studied *Tilletiopsis* spp., especially *T. minor*, on cucumber powdery mildew (Hijwegen, 1986, 1988). *Tilletiopsis* spp. are ubiquitous inhabitants of the phyllosphere worldwide. Virtually every mature leaf carries *Tilletiopsis* colonies. However, powdery mildew-parasitizing strains may belong to specialized groups, since all *T. pallescens* isolates from powdery mildew had

similar DNA patterns different from other isolates of the same species (Boekhout et al., 1992).

Humidity plays a decisive role in mycoparasitism. Most, if not all, mycoparasites require high humidity for activity, as has been demonstrated by various authors. Reduction of humidity, as is usual under prevailing greenhouse conditions, will result in lowered effectivity. With *T. minor*, results of treatments at a relative humidity below 80% did not reach an acceptable level of powdery mildew control. This loss of activity could be counteracted by the addition of 0.1-0.5% Hora Oleo 11E, a formulated paraffine-oil (Hijwegen, 1992b). Then results were obtained comparable to those with fungicide. In three pot experiments yields of marketable cucumbers of the treatments with *T. minor* + paraffine-oil and with fungicide did not differ significantly ($P < 0.05$).

In summary, it can be concluded, that rather good control of cucumber powdery mildew can be obtained with formulated *T. minor*. Nevertheless, it is a rather weak mycoparasite. Therefore we turned to a more agressive fungus, *Verticillium lecanii*, which shows great promise for biological control. Moreover, another strain of this fungus has a registration in the Netherlands and the United Kingdom for the control of whiteflies in greenhouse cucumber production. This could be advantageous in the case of clearance procedures. Experiments with *V.lecanii* are discussed in this volume by Verhaar and Hijwegen.

2. Downy mildews.

Little is reported in the literature on above-ground mycoparasites of downy mildews (Peronosporales). Surprisingly, we encountered the same fungi as on powdery mildews in a limited survey, *V. lecanii* being the most abundant. Against *Peronospora parasitica* on red cabbage the best results were obtained with *Aphanocladium album* and *V. lecanii*, both giving 95% parasitation after 7 days. The same fungi were the best parasites of *Phytophthora infestans* grown in petri dishes.

Oomycetes, such as *Peronospora* and *Phytophthora*, are known as "cellulose fungi". They are supposed to have cellulose in their cell walls. However, Jarvis (1988) studying cell walls of *Phytophthora infestans* by means of NMR spectroscopy concluded that the main component was ß-(1,3)-glucan. He could not demonstrate the presence of cellulose or chitin. Probably, also against Oomycetes the capacity to degrade ß-(1,3)-glucan is the most important feature.

Finally some remarks on induced resistance. When fungicides are banned and we have to rely on alternative methods, the approach should be three-fold in our opinion:

1. Choose varieties that are at least partially resistant (when available).
2. Control with biological means.
3. Hope, that the plant will also help by providing some induced resistance.

To test the last possibility we studied inducible resistance in red cabbage. We found that one application of 2-4 ppm 2,6-dichloroisonicotinic acid (INA: CGA 41396, Ciba-Geigy AG) at 4 days before inoculation gave (almost) complete control against *P. parasitica*. We studied two varieties and found that the variety with the highest partial resistance also had the highest inducible resistance.

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BIOLOGICAL CONTROL OF CUCUMBER POWDERY MILDEW (*SPHAEROTHECA FULIGINEA*) BY VERTICILLIUM LECANII AND *SPOROTHRIX* CF. *FLOCCULOSA*.

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Summary

Two glasshouse experiments were done to test biological control of powdery mildew in combination with partial resistance. The infected leaf area on the partially resistant cucumber cultivar Flamingo could be kept under the economic threshold by the use of treatments with *V. lecanii*.

Introduction

Several mycoparasites of cucumber powdery mildew (*Sphaerotheca fuliginea*) have been described (Philipp, 1988; Burge, 1988; Hijwegen, 1988). Usually, experiments are done with susceptible varieties. We hypothesised that partially resistant varieties perform better. On partially resistant cucumber cultivars the mildew grows slowly, which gives the mycoparasites a better opportunity to destroy its colonies (Verhaar, unpublished).

Two glasshouse experiments were performed to test the combination of biological control of powdery mildew with the use of partially resistant cucumber cultivars. As biological control agents two mycoparasites, with high potential in the in vitro tests (Verhaar, unpublished), *Verticillium lecanii* and *Sporothrix* cf. *flocculosa*, were used.

Materials and methods

Experiments were performed with the susceptible cucumber cultivar Corona and the partially resistant cultivar Flamingo. Three weeks old plants were planted in soil in a glasshouse. When the plants were six weeks old leaves seven and eight were inoculated with powdery mildew in water(10^5 spores/ml). Two weeks after the inoculation the biological control treatments were started.

Plants were sprayed with $5*10^6$ spores/ml of *V. lecanii* or *S.* cf. *flocculosa*. The percentages leaf area covered with mildew were assessed weekly using a ten class key. The middle and top leaf levels were rated.

In the second experiment treatments with *V.lecanii* or water (as a control) were applied at one and two week intervals on Flamingo plants inoculated with powdery mildew.

Results

Table 1 shows the average mildewed area two months after the inoculation with powdery mildew in experiment I. The results of four treatment combinations of cultivars and mycoparasites are compared. There is a remarkable difference between the cultivars with respect to mildew development. On both cucumber cultivars *V. lecanii* restricted the mildewed leaf area to about one half of that in the *S.* cf. *flocculosa* treatment.

Table 1.

TREATMENTS fungus	cultivar	MEANS *) percentage infected leaf area	MEANS *) after arcsin transformation
V. lecanii	Flamingo	14.3	19.8 a
S. flocculosa	Flamingo	27.7	30.7 b
V. lecanii	Corona	41.4	35.8 b
S. flocculosa	Corona	77.6	61.3 c

*) average of six treatments
Means followed by different letters are significantly different (LSD=11.2, P<=0.05).

In experiment II good results were obtained in both *V. lecanii* treatments. The greatest differences were found between *V. lecanii* once a week and a water treatment every two weeks. These are shown in figure 1.

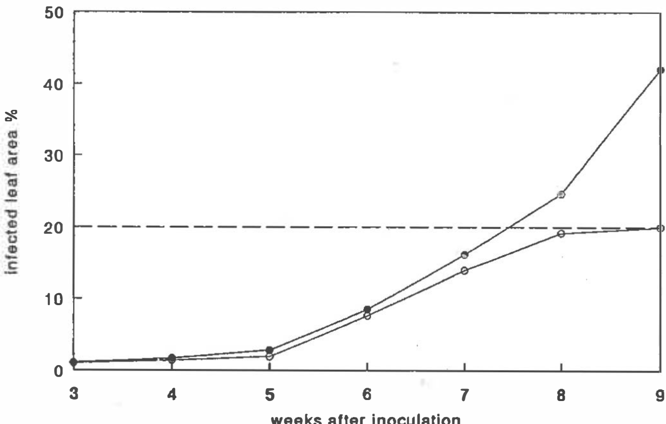

Fig. 1. Experiment II. Mildew development on cucumber, cv. **Flamingo**, with different control treatments. The open circles represent the *Verticilium lecanii* treatment once a week and the closed circles represent the water treatment every two weeks. The dotted line indicates the 20% economic threshold.

Discussion

Under glasshouse conditions *V. lecanii* gives better control of powdery mildew than *S.* cf. *flocculosa*. Climatic conditions have effects on the activity of mycoparasites. Especially humidity and temperature are important (Hajlaoui and Bélanger, 1991). Formulations can be used to enhance the microclimatic conditions (Philipp et al., 1990; Hijwegen, 1992). Formulation of mycoparasites in the first glasshouse experiment improved the results. (Verhaar, unpublished).

Biological control of mildew on the partially resistant cucumber cultivar Flamingo gave good results. In both experiments we could keep the mildew infected leaf area below the 20% economic threshold by the use of a weekly

V. lecanii treatment. During the experiments there was a high mildew inoculum pressure. Without this inoculum pressure the biological control by *V. lecanii* might have given even better results.
We suppose that in the future a combination of partially resistant cucumber cultivars with biological control will be successful.

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BIOLOGICAL CONTROL OF POWDERY MILDEW WITH FUNGAL ANTAGONISTS: COMPARATIVE EFFICACY AND MODE OF ACTION.

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Abstract

Three reported antagonists of cucumber powdery mildew, *Stephanoascus flocculosus, St. rugulosus,* and *Tilletiopsis washingtonensis,* were tested and compared under different environmental conditions for their potential for controlling rose powdery mildew, caused by *Sphaerotheca pannosa* var. *rosae*. Both temperature and relative humidity (r. h.) affected the activity of the antagonists differently. The colonization of powdery mildew was maximal at 26°C, especially for *St. rugulosus* and *T. washingtonensis*. Maximal colonization was achieved at the highest r.h. tested (90%) for all three antagonists but only *St. flocculosus* maintained a colonization of 80% or better under lower r.h. Under large-scale conditions, the latter antagonist was able to control powdery mildew only if the humidity conditions prevailing in the experimental area were appropriate. Ultrastructural observations revealed that antibiosis rather than chitinolytic activity was involved in the antagonistic process against *S. pannosa* var *rosae.*

Introduction

Powdery mildew of roses caused by *Sphaerotheca pannosa* var. *rosae* is the single most important disease of greenhouse roses. In spite of recent developments in the biocontrol of powdery mildews, few if any antagonists have been tested against *S. pannosa* var. *rosae*. However, for the powdery mildew fungi, the existence of hyperparasites and antagonists has been clearly established. For example, species of *Tilletiopsis* have been reported to control *Sphaerotheca fuliginea* (cucumber powdery mildew) (Hoch and Provvidenti, 1979; Hijwegen, 1986, 1988), *Erysiphe graminis* var. *hordei* (barley powdery mildew) (Klecan et al., 1990) and powdery mildews of other plant species (Hijwegen and Buchenauer, 1984). More recently, two newly identified fungi, *Stephanoascus flocculosus* and *St. rugulosus* were found to colonize and inactivate *S. fuliginea* on cucumber. It was further shown that the activity of these antagonists was greatly influenced by environmental conditions, especially relative humidity (Jarvis et al., 1989). In light of these observations, the objectives of this study were 1) to determine the effects of temperature and humidity on the activity of *St. flocculosus, St. rugulosus* and *T. washingtonensis* against rose powdery mildew and 2) to study at the ultrastructural level the antagonistic process by which these antagonists could attack *S. pannosa* var *rosae*.

Results

1. Effect of temperature. The best colonization occurred at 26 °C with *St. flocculosus* achieving complete overgrowth in approximately 48 h and *St. rugulosus* anf *T. washingtonensis* in 5 days (Fig. 1). Complete colonization was also achieved at 30 °C in 5 days by *St. flocculosus* while a colonization of 80% was noted after 3 and 7 days at temperatures of 22 and 34 °C respectively (Fig. 1A). For *St. rugulosus* and *T. washingtonensis* temperatures below 22 °C and above 30 °C achieved less than 50% colonization of *S. pannosa* var. *rosae* (Figs. 1B-C). At any given temperature, *St. flocculosus* achieved a higher rate of colonization than the other two antagonists.

2. Effect of humidity. In growth chambers, only a r.h. of 90% achieved complete colonization of *S. pannosa* var. *rosae* by all three antagonists (Fig. 2). Again *St. flocculosus* exhibited a faster activity than the other two antagonists tested. The superior activity of *St. flocculosus* was also noticeable at r.h. 80% and 70% where it reached complete overgrowth within 7 days at r.h. 80% and 80% colonization at r.h. 70% (Fig. 2A). By contrast, *St. rugulosus* and *T. washingtonensis* never attained maximum colonization below r.h. 90% with the latter being the least active especially at r.h. 70% (Figs. 2B-C).
Under large-scale conditions, control of rose powdery mildew was as effective with applications of the antagonists as with fungicides when relative humidity conditions were maintained constant with a misting system. There was a loss of efficacy when humidity conditions dropped sharply. However, control could be restored if the r.h. level was brought back to a higher level. The quality of the plants was not affected by the biological treatment with the antagonists.

3. Mode of action. Scanning and transmission electron microcopy investigations of the interaction between the antagonists and *S. pannosa* var *rosae* revealed that all three fungi induced a rapid collapse of conidia, conidiophores and hyphae of the host fungus within 24 h following their application. Observations using a wheat germ agglutinin/ovomucoid-gold complex as a specific probe for localizing chitin distribution in cells of the rose powdery mildew fungus, revealed that close contact between the interacting fungi was rarely associated with the penetration of the host cells by the antagonist but that considerable changes in the cytoplasm with no discernible alteration of the chitin labeling distribution over the cell walls could be observed. After 48 h, the antagonists caused complete plasmolysis of host hyphae which were reduced to only cell walls. These results, based on ultrastructural observations and cytochemical localization of N-acetylglucosamine, suggest that rather antibiosis than chitinolytic activity is involved in the antagonistic process of the tested antagonists against *S. pannosa* var. *rosae*.

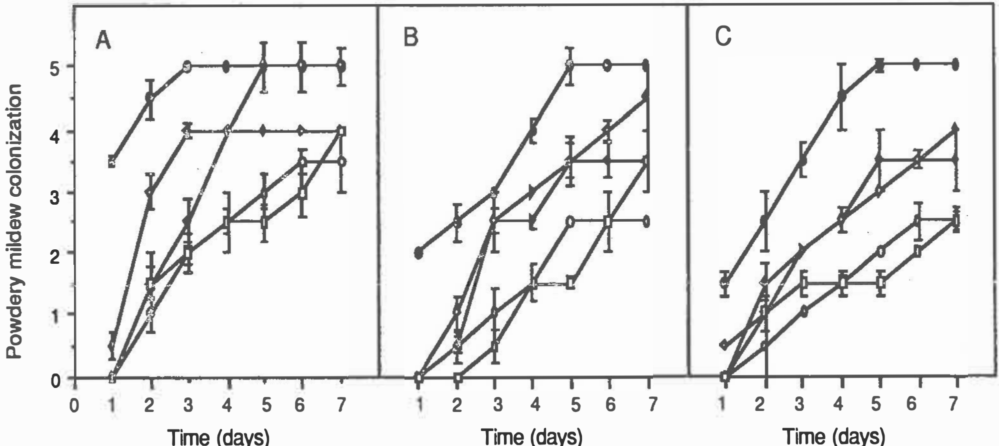

Fig. 1. Effect of temperature on colonization of Sphaerotheca pannosa f. sp. rosae by **A**. Stephanoascus flocculosus, **B**. St. rugulosus, and **C**. Tilletiopsis washingtonensis. O-O = 18°C, ◈-◈ = 22°C, ●-● = 26°C, ◇-◇ = 30°C, and □-□ = 34°C. Vertical bars represent standard error of the mean. Relative humidity was set at 90%.

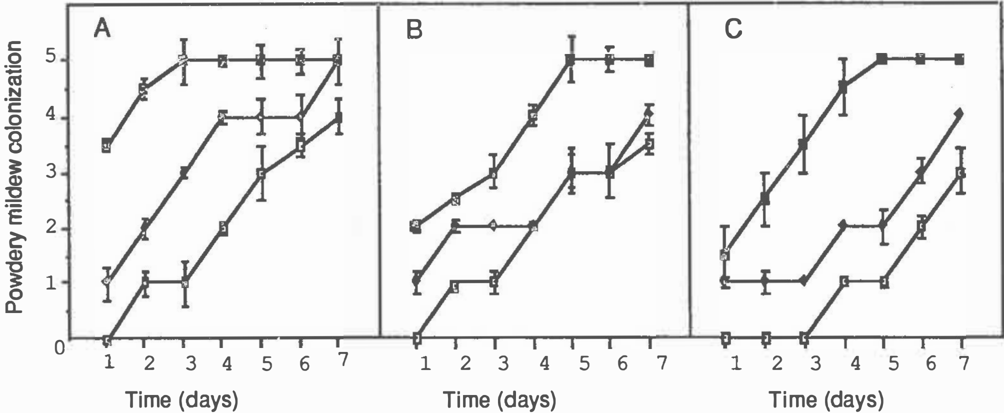

Fig. 2. Effect of relative humidity on colonization of Sphaerotheca pannosa f. sp. rosae by **A**. Stephanoascus flocculosus, **B**. St. rugulosus, and **C**. Tilletiopsis washingtonensis. ▣-▣ = 90%, ◈-◈ = 80%, and ▣-▣ = 70%. Vertical bars represent standard error of the mean. Temperature was set at 25 °C.

Reproduced from Hajlaoui & Bélanger, 1991, with permission from Netherlands Journal of Plant Pathology

Discussion

Our results clearly indicate that *St. flocculosus, St. rugulosus* and *T. washingtonensis* can impede the development of *S. pannosa* var. *rosae*. Although all three fungi tested were shown to be antagonistic to rose powdery mildew under *in vitro* conditions, tests on mature plants revealed that the activity of the antagonists was considerably decreased under low ambient humidity conditions and temperatures outside a range of 22 to 30 °C. These observations stress the importance of considering environmental conditions when assessing the activity of antagonistic microorganisms.
Involvement of enzymes in biocontrol blurs the distinction between parasitism and antibiosis. In our study, the absence of wall dissolution associated with a rapid collapse of host hyphae leads to speculation on the importance of antibiosis in the antagonistic activity of the tested organisms. Antibiosis can be considered as providing advantages over other types of antagonism for biological control because antibiotic substances may diffuse rapidly, and thus direct contact between the two partners is not necessary. Our ultrastructural observations reveal that the need for contact or presence in close proximity of the antagonist is not essential before the induction of a harmful effect. A similar mode of action has been previously reported as a general feature of necrotrophic parasitism in which lytic enzymes and toxic metabolites are implicated with, but, a more important role ascribed to antibiosis (Howell, 1987).

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IMPROVEMENT IN THE HYPERPARASITIC ACTIVITY OF *AMPELOMYCES QUISQUALIS* IN THE BIOCONTROL OF POWDERY MILDEW OF CUCUMBER

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Summary

Mutant strains of *Ampelomyces quisqualis*, which grew on media of which the water activity had been reduced, germinated at lower relative humidities than wild-type strains. These mutant strains were more effective than wild-types in reducing mildew infection of cucumber plants and suppressing sporulation by *Sphaerotheca fuliginea* on cucumber leaves in glasshouse trials.

Introduction

Hyperparasitism of *Sphaerotheca fuliginea* on cucumber by *Ampelomyces quisqualis* is dependent on the presence of liquid water and requires the maintenance of high levels of relative humidity (r.h.) (Philipp and Crüger, 1979). This requirement results in heavy dependence on environmental factors and decreases the likelihood of effective biological control of powdery mildew by the hyperparasite. Consequently, it was decided to attempt to reduce this humidity requirement in the hope of increasing the biocontrol capability of *A. quisqualis*.

Materials & Methods

Mutants produced by exposure of pycnidiospores of an isolate of *A. quisqualis* to short-wave UV light were selected by their ability to grow on V-8 juice agar of which the water activity (a_w) had been reduced by the addition of polyethylene glycol or glycerol. The formation of colonies on these media indicated tolerance to low a_w for germination and mycelial growth. Water activity is numerically equivalent to the corresponding r.h. expressed in decimal form; i.e. tolerance of 0.94 a_w implies an ability to germinate and grow at 94% r.h.
The mutant strains of *A. quisqualis* were compared with the wild-type isolate A for germination and radial growth rate on reduced a_w media and at a range of r.h. between 60% and 100%. Using cucumber plants infected with powdery mildew, strains were compared for their effect on mycelial density and conidiation of *S. fuliginea*, under a range of r.h. The ability of the *A. quisqualis* strains to reduce the rate of mildew infection, and their effect on fruit yield in a glasshouse trial, were assessed in comparison with a standard fungicide treatment.
When *S. fuliginea* was inoculated with *A. quisqualis* on cucumber leaves, because of the possible effect on mildew development of the water in which the *A. quisqualis* inoculum was suspended, a mildew-infected control treatment was sprayed with sterile water at the time of inoculation of other treatments with the hyperparasite (wet control). A further mildew-infected control remained dry (dry control).

Results & Discussion

Three mutant strains of A. quisqualis, designated M9215, M9415 and M9417, germinated and grew profusely on V-8 agar of 0.946 a_w and sparingly at 0.928 a_w, whereas the wild-type isolate (A) from which these mutants were derived, and a number of other wild-type isolates (B, C & D) would not germinate below 0.95 a_w (Fig. 1). Radial growth rates of the mutants and the wild-type isolates on unamended V-8 agar were not significantly different.

Fig. 1 Effect of reduced water activity on germination of wild isolates of Ampelomyces quisqualis

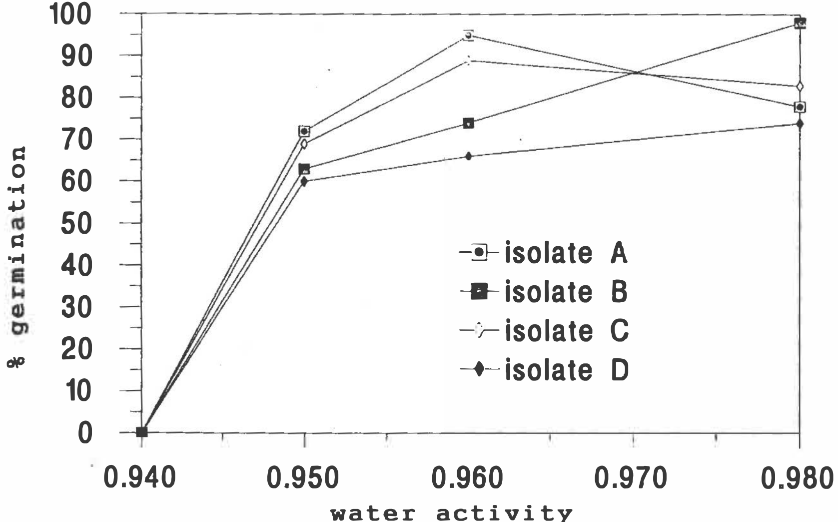
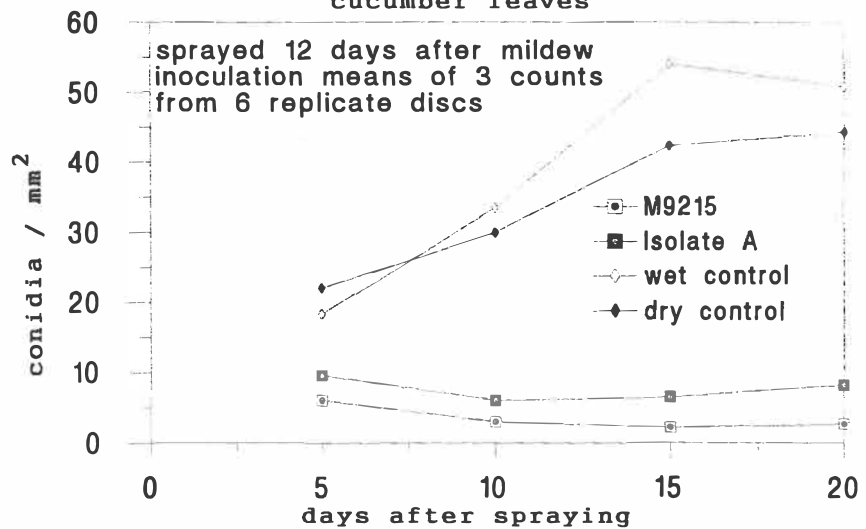


Fig. 2 Effect of Ampelomyces quisqualis strains on conidium production by Sphaerotheca fuliginea on cucumber leaves



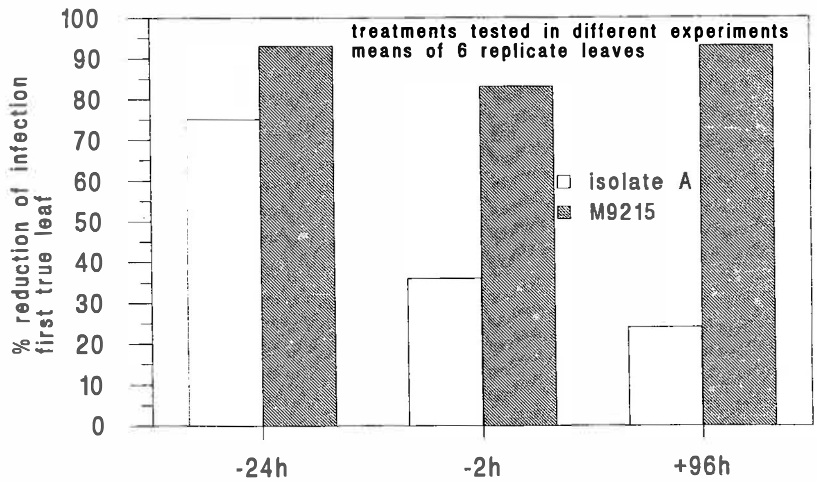
Pycnidiospores produced by the mutant strains germinated more readily than isolate A at 60, 70, 80, 90 and 95% relative humidity (r.h.), although the length of germ tubes produced in 72h at these humidities did not differ significantly between mutant strains and the wild-type, suggesting that reduced humidity has a more inhibitory effect on spore germination than on mycelial growth. Germination of M9215 was significantly higher than the other mutants at 80 and 90% r.h.

Two mutant strains were compared with isolate A for their effect on conidium formation by S. fuliginea, on leaves of cucumber plants growing in a growth chamber at 80% r.h. Both mutants had a significant effect on conidiation, and one strain (M9215) reduced conidium production significantly better (94%) than isolate A (83%) 20 days after A. quisqualis inoculation (Fig. 2).

With the wild-type isolate A, prior inoculation (-24h) of cucumber leaves with A. quisqualis was more effective in reducing the number of mildew lesions formed after infection with S. fuliginea than "simultaneous" (actually -2h) or post-infection (+96h) inoculation. However, this distinction was much less with the mutant strains, frequently exhibiting similar levels of control whether applied 24h before, or 96h after, mildew inoculation (Fig. 3).

In a glasshouse trial at a mean r.h. of approximately 65%, while not as effective as a fungicide treatment (0.2% nimrod), the mutant strain M9215 reduced the area of leaf from which S. fuliginea conidia were produced (Fig. 4), and reduced leaf loss and numbers of conidia formed more than the wild-type isolate (Table 1). The yield of cucumber fruit was also substantially higher when plants had been treated with the mutant strain compared with the effect of isolate A (Table 1).

Fig. 3 Effect on powdery mildew infection of various sequences of inoculation with Ampelomyces quisqualis



time of A. quisqualis inoculation relative to mildew infection

Fig. 4 % leaf area of conidiophore suppression

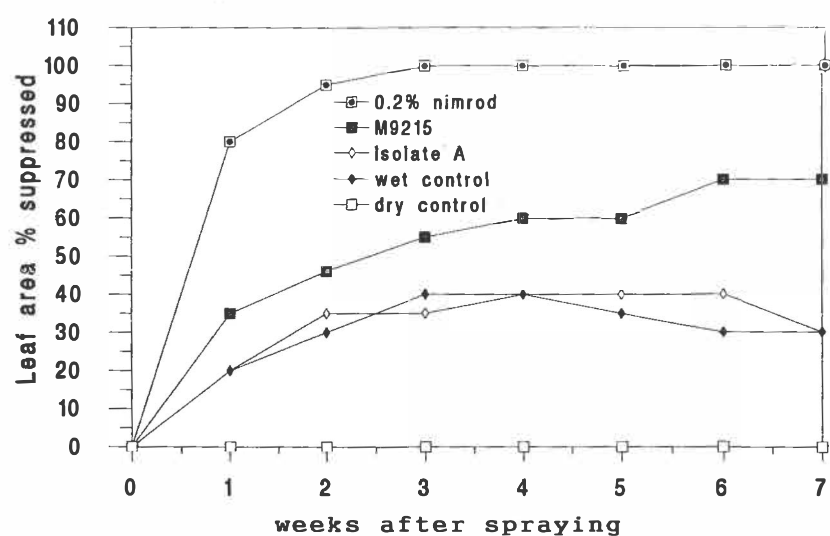


Table 1. Effect of A. quisqualis and fungicide on control of powdery mildew infection on cucumber.

Treatment	No. dead leaves*$ (SD)	Conidia ($x10^5$)/ cm^2 leaf#$ (SD)	Total yield of 12 plants (kg)
0.2% nimrod	0.0	0.0	3.2
dry control	8.25(1.0)	5.70(1.9)	1.0
wet control	7.30(0.9)	4.20(1.5)	1.2
M9215	5.70(0.8)	0.95(1.0)	2.0
isolate A	6.40(0.6)	2.20(2.1)	0.8

* dead leaves on lowest metre of stem, mean of 12 plants
\# mean of 3 counts from 6 replicate leaf discs (leaf 12)
$ assessed 7 weeks after start of spray programme

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PERSPECTIVES FOR THE FUTURE: CONTROL OF BIOTROPHS BY MYCOPARASITES

SUMMARY OF THE DISCUSSION

KURT MENDGEN

The biological control of biotrophic fungi is difficult, but not hopeless. Excellent examples for the powdery mildew fungi can be found in the reports by Hijwegen and Bélanger's group.

A major improvement of biological control may be obtained by improving the isolates of the microorganism used. The screening procedures for better isolates should include methods that improve the ability of the biological control agent to grow under lower humidity conditions. The vapor pressure deficit under which the fungus is able to grow on agar or within the pustule of the pathogen should be studied more carefully .

A major problem is the formulation of the biological control agent. Especially the control of powdery mildew fungi was considerably improved by the use of mineral oil. For the future, the use of vegetable oils is recommended. It was generally suggested to increase research on formulation of control agents because very few results are published on this subject.

Besides fungi as biological control agents, plant extracts, bacterial metabolites or compost extracts may be used to protect plants against many fungal pathogens. The active compounds of such extracts are still unknown. They possibly activate defence mechanisms of the parasitized plant. The disadvantage of this way of control is that it has to be applied several days before the pathogen arrives and protection lasts only for few days. A combination of different methods, such as fungi and extracts may be useful because they can match diverse conditions in the field.

The small markets for biological control agents still pose a major obstacle to their commercialisation. However, this may change because of increased problems with fungicide resistance and the awareness of consumers of residues on fruits and vegetables. Since the development of a system for biological control takes at least 6 - 8 years, the participants of the symposium felt that we have to increase our research efforts in order to have optimized methods available in time. In the near future, market chances may increase dramatically and by this time, results and methods that can be used under practical conditions, should be ready.

A mixture of biological control agents with fungicides is not recommended. It is doubtfull whether this is suitable for the application and for the reputation of biological control. Also, it may have a negative effect on the waiting time required before consuming.

Biological control of post-harvest diseases

BIOLOGICAL CONTROL OF POSTHARVEST DISEASES - INTRODUCTION

EDO CHALUTZ, The Volcani Center, Israel.

Welcome to the first session in our workshop devoted to postharvest matters. The idea of including in this workshop aspects of biological control of both pre and postharvest diseases has proven so far as a well justified approach.

This field, the biological control of postharvest diseases, is a relatively new research area. While some publications can be found in the literature as early as the mid 50's, mostly dealing with the biocontrol activity of *Bacillus subtilis*, the main thrust of the research has been initiated in the 80's. Charlie Wilson, of the USDA, Appalachian Fruit Research Station in Kearneysville, West Virginia, U.S.A. is to be credited for this renewed effort. Since then, an increasing number of scientists throughout the world turned to explore the potential of this new technology, with a few private firms following.

In 1988, the International Congress o Plant Pathology allocated, for the first time, a session to discuss biological control of postharvest diseases, while commemorating "50 years of biological control research" in other fields. Since then, however, at least one international workshop has been devoted entirely to discussing biological control of postharvest diseases and, in the forthcoming meeting of the International Congress of Plant Pathology, to be convened in Canada next summer, this topic will receive considerable attention.

Why this sudden surge in activity on biological control of postharvest diseases? The main reason is, no doubt, the mounting pressure by health authorities and consumers alike to reduce the use of synthetic chemical fungicides, particularly on food products. Not only has this pressure resulted in the banning (or voluntarily withdrawing from the marked of important fungicides used to protect fruits and vegetables from postharvest rots, but it has also lead to a re-evaluation of the safety of many fungicides currently in use. A statement suggesting that a committee of the European Community has recommended to ban all postharvest use of chemical fungicides "as soon as this practice becomes feasible" has also been reported.

Whether or not all aspects of this new attitude are scientifically sound or justified is not clear. Nonetheless, is has raised, for the first time, the real possibility that some fruits and vegetables will be left without the means to protecting them from decay after harvest.

On this basis, therefore, there seem to be a full justification for the increasing efforts by the scientific community to find alternative means to the use of the chemical fungicides - biological or others - that are safe to human health and the environment. Similarly private firms, which in the past did not always consider it worthwhile to invest in the postharvest decay control market because of its relatively small size, are now more willing to do it.

Some two years ago we prepared the following two slides to show the locations in the U.S. and Europe where research on biological control of postharvest diseases was taking place. Today, many other site would have been added to the map, as can be learned from the contributors to this workshop. Several private firms have invested resources and are currently conducting large scale tests to develop the knowhow and the data base needed for formulating and licensing biological control agents of postharvest diseases for commercial use.

Will we have to wait another 40 or 50 years for the implementation of the results of these efforts? Probably not. Firstly because we simply do not have that much time. But also we may have chosen the right concept by using naturally occurring, rather than genetically or otherwise modified antagonist, which are much more likely of being approved for use of fresh commodities than the altered ones. Most important, however, we may not have to wait so much time because the postharvest environment seems to offer a better chance of success for biological control than do the field or soil environments: the controlled temperature and humidity, the restricted and well-defined site of application of the biocontrol agent, the relatively high price of the harvested fruit or vegetable and the fact that protection is sometimes need for only a short time,are all increasing the chances of success. On the other hand, the fact that we may be adding large numbers of microorganisms directly to food products, the very high and consistent efficacy required and the relatively small potential market are hindering factors that we have to overcomed.

Judging from the topics of the presentations of today's session, as well as from the literature, one may conclude that a wide variety of diseases on different crops are currently investigated with regards to biological control of postharvest diseases.

Quite noticable, however, is the fact that the antagonists investigated are, in many cases, naturally occurring yeasts. This may not be a coincidence. The yeast microorganisms posses characteristics that may make them particularly suitable as antagonists. These include the following features:

- rapid growth on the commodity, thus successfully competing with the pathogen;
- persistent on the commodity for long periods - prolonged protection effect;
- resistant more than most postharvest pathogens to extreme environmental conditions such as temperature, humidity, pH, gas composition, and osmotic conditions;
- resistance to chemicals, including fungicides, thereby enabling the development of integrated control approaches;
- compatible with postharvest processing procedures;
- not pathogenic to most commodities;
- not fastidious in nutrient requirements - can be cultured on cheep media;
- can be formulated to a product with a long shelf life;
- few produce antibiotics or other metabolites that may be dangerous to human health;
- some do not grow at or above human body temperature and therefore are less likely to pose health hazards.

Let us turn now to the presentations of the talks with the hope that some of the topics touched on during this introduction will be discussed at the end of the session.

BIOLOGICAL CONTROL OF BOTRYTIS ROT OF KIWI FRUIT *

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Summary

Some microrganisms isolated from untreated fruit showed an interesting biocontrol activity against Botrytis rot of kiwi fruit. The activity of some of them was confirmed when they were applied by dipping in a 10^7 cells/ml suspension under semi-commercial conditions, in the presence of attacks of *Botrytis cinerea* of medium intensity.

1. Introduction

Postharvest losses due to Botrytis rot can be very serious on kiwi fruit - an important crop in Italy - even where the most advanced storage facilities are available. The control of *Botrytis cinerea* on kiwi relies on field sprays with dicarboximides, unless postharvest treatments are admitted. Although postharvest treatments - not admitted on kiwi in Italy - generally show the best efficacy (Bisiach and Minervini, 1984; Ponti *et al.*, 1990), they leave residues higher than those admitted (Tonini *et al.*, 1992). Moreover, strains of *Botrytis cinerea* resistant to dicarboximides have been detected in some production areas.
Since the search for control measures alternative to chemicals looks particularly promising (Wilson *et al.*, 1991), our work was aimed to select microrganisms active against Botrytis rot of kiwi under semi-commercial conditions.

2. Materials and methods

Several microrganisms, most bacteria and yeasts, were isolated from the surface of kiwi fruit harvested in unsprayed orchards by following a procedure already reported (Gullino *et al.*, 1991). Bacteria were maintained on Nutrient Yeast Glucose Agar (NYGA), yeasts on malt agar: all cultures were coded and stored at 6-8 °C until tested on fruit. Also, two yeasts, previously

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* Work partially carried out with a grant to DI.VA.P.R.A. from MURST (40%: Nuove strategie di difesa delle piante a basso rischio ambientale).

described for their activity against Botrytis and Penicillium rots of apple (Gullino _et al._,1992), were used.
Biocontrol activity of several isolates was tested on fruit (5 replicates/isolate) wounded at the peduncle level and treated by dipping for 30' in 10^7 cells/ml suspensions of each candidate. Fruit were maintained 24 hours at 20-22 ^oC, then inoculated by dipping for 10' in a 10^6 conidia/ml suspension of a mixture of at least 4 strains of _Botrytis cinerea._ Fruit were kept for 15 days at 2 ^oC, then transferred at 20-22 ^oC for 6 days. All isolates have been tested at least twice.
Microrganisms showing the highest activity were used in larger scale and semi-commercial conditions. In the first case, 10% of kiwi were wounded at the peduncle level, then dipped for 30' in 10^7 cells/ml of the selected antagonist or mixture of antagonists. After 24 hours at 20-22 ^oC, they were dipped in a 10^6 conidia/ml suspension of a mixture of strains of _B.cinerea_ sensitive (S) or resistant to both benzimidazoles and dicarboximides (RBD). Fruit were kept at 0 ^oC and 90% RH for 25 days, transferred for 5 days at 20-22 ^oC. Forty fruit/ treatment were used. When operating under semi-commercial conditions, inoculum of _B.cinerea_ was natural and 160 fruit/treatment were used. Also in this case, biocontrol candidates were applied, immediately after harvest, by dipping fruit in 10^7 cells/ml suspensions. Two different intervals, (0 and 96 hours), between treatment with the antagonist and transfer at 0 ^oC were tested. The fungicides used, as commercial formulations, are reported in tables 2 and 4. Fruit belonging to the cv Hayward have been used. Rot was evaluated after cutting in half the fruit; considering that it always was very extensive, data are expressed as % healthy fruit.

3. Results

Nine of the tested microrganisms - 4 yeasts and 5 bacteria - did show a satisfactory biocontrol activity against Botrytis rot, giving at least a 50% reduction of losses (table 1). When tested on a larger scale, _Candida_ sp. isolate 4.4 showed the best activity as compared with _Trichosporon_ sp. and two mixtures of microrganisms, selected after the screening. The fungicides TBZ and vinclozolin controlled Botrytis rot only when the pathogen was sensitive to both groups of fungicides (table 2). Both _Trichosporon_ sp. and _Candida_ sp. confirmed their good activity under semi-commercial conditions, in the presence of a natural infection. A delay before refrigeration, indicated by some authors as positive against Botrytis rot, did not reduce damage under our experimental conditions (Brigati _et al.,_ 1992). However, a longer interval between treatment with the antagonist and refrigeration did sometimes sligthly improve the antagonist performance, probably by allowing a better colonization of the fruit (table 3).
In conclusion, although much still needs to be done in order to improve the activity of the selected microrganisms, also through formulation, the possible alternatives to chemical control looks particularly promising in the case of a crop where postharvest treatments are not permitted.

Table 1 - Activity of different microrganisms against Botrytis rot of kiwi.

Isolate	% healthy fruit	Isolate	% healthy fruit	Isolate	% healthy fruit
--	10	Y K 3	20	B K 24	20
B 1.74	40	B K 4	70	Y K 25	30
B 1.62	60	Y K 5	60	B K 26	0
B 2.97	50	Y K 6	70	Y K 27	20
Y 1.52	10	Y K 7	10	B K 28	0
Y 2.55	30	Y K 8	0	B K 29	30
B 1.85	29	Y K 9	60	B K 30	30
Y 2.52	40	B K 10	10	Y K 31	20
Y 4.4	23	Y K 11	70	Y K 32	20
Y 2.61	40	B K 12	60	B K 33	20
B 2.59	20	Y K 13	0	Y K 34	0
B 1.89	20	Y K 14	10	B K 35	10
B 2.29	30	B K 15	30	B K 36	20
Y 2.100	20	B K 16	20	B K 37	20
Y 1.48	50	B K 17	0	B K 38	0
B 7.115	0	Y K 18	20	Y K 39	0
Y 1.46	25	B K 19	0	B K 40	0
Y 2.33	34	B K 20	10	Y K 41	0
B 2.43	34	B K 21	30	B K 42	10
Y K 1	10	B K 22	40	B K 43	0
Y K 2	0	B K 23	20	B K 44	0

Table 2 - Effectiveness of different treatments against Botrytis rot of Kiwi, incited by strains of B.cinerea sensitive (S) or resistant to benzimidazoles and dicarboximides (RBD).

Treatment *	% healthy fruit after inoculation B. cinerea S	B.cinerea RBD
Control, not inoculated	93 a **	93 a
Control, inoculated	60 b	68 b
Trichosporon sp. 2.33	75 ab	75 ab
Candida sp. 4.4	87 a	81 ab
Mixture K1	77 ab	73 b
Mixture K2	75 ab	72 b
TBZ 1 g/l	96 a	61 b
Vinclozolin 1 g/l	95 a	65 b

* Treatment with antagonists carried out by dipping in 10^7 cells/ml. Mixture K1: isolates K4+K5+K6; K2: K9+K11+K12

** Values followed by the same letter do not statistically differ (P = 0.05) following Duncan's Multiple Range Test.

Table 3 - Effectiveness of different treatments against Botrytis rot of kiwi under semi-commercial conditions.

Treatment *	Hours before ** refrigeration	% healthy fruit***
--	0	88 b ****
--	96	58 c
Trichosporon sp. 2.33	0	96 a
Trichosporon sp. 2.33	96	100 a
Candida sp. 4.4	0	95 a
Candida sp. 4.4	96	97 a
Vinclozolin (0.75 g/l)	0	97 a
Vinclozolin (0.75 g/l)	96	97 a

* Treatments were carried out on October 29, 1991. Yeasts were applied by dipping in 10^7 cells/ml suspension
** Interval between treatment and refrigeration
*** Evaluated on February 11, 1992
**** See table 2

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BIOLOGICAL CONTROL OF POST-HARVEST DISEASES ON CARROTS.

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Summary

Storage rot on carrots caused by *Botrytis cinerea, Mycocentrospora acerina, Rhizoctonia carotae* and *Sclerotium sclerotiorum* was reduced during long term storage at 0°C by dipping the roots after harvest in a conidial spore suspension of the antagonistic fungus *Trichoderma harzianum* P1.

Introduction

Biological control of soil borne and foliar diseases has been in the focus of interest for a long time, but little interest has been focused on control of crop after harvest (Cook & Baker 1983). In 1985, however, Wilson & Pusey (1985), pointed out that there should be good possibilities for control of post harvest diseases of three reasons. 1. It is possible to control the storage environment. 2. It is easy to target the application to the product to be protected. 3. Caused by the high value of the harvested crop, rather elaborate control procedures, that may not be economical feasible under field conditions, are cost-effective for harvested food.

However, even if the potential for biological control of post-harvest diseases is good, few results so far have been published where realistic storage conditions and storage times have been applied. One of the main reasons is that the climatic conditions best suited for the crop, often close to 0°C, are not suitable for the growth and antagonistic activity of most biocontrol agents. Changing the conditions in favour of the antagonist and not the crop, is not a solution since that will cause new and often severe problems. Therefore, to obtain a successful biological control, antagonists are needed that can perform control at the conditions best suited for storage of the crop.

The demand for cold tolerant antagonists limits the amount of possible biocontrol agents, as most isolated antagonists do not have

any significant activity below 10°C. Work with antagonistic isolates of Trichoderma spp. however, has showed that it is possible to select isolates that act antagonistically at low temperature (Tronsmo & Dennis 1978). One of these isolates has been used in control of storage rot on carrots.

Materials and methods.

The fungal antagonist used was Trichoderma harzianum Rifai P1 (ATCC 74058) (Tronsmo 1989). Carrots (Daucus carota L.) were harvested two years from two fields in Norway, one field heavy naturally infected by Mycocentrospora acerina Hartig, and the other naturally infected by Rhizoctonia carotae Rader. No fungicides were used, but chemical control of pests and weeds was carried out. At harvest the carrots were placed in polyethylene nets (12kg/net) and stored in 400kg bins where the bottom, sides and top were covered with polyethylene to prevent the carrots from drying out. Room temperature during the storage period was 0-0.5°C, but it took 2 months for the temperature in the middle of the produce to come down to 2°C, where it stabilized for the rest of the storage period. The biological control treatment was performed before storage by soaking the nets in a conidial suspension (10^7 conidia/ml) of T. harzianum P1. The treatment caused an average surface coverage of 10^3 cfu T. harzianum P1 per cm2 (Tronsmo & Hoftun 1984). At each registration, three bags of each treatment were examined and divided into the following groups: No visible disease symptoms, rot caused by Botrytis cinerea Pers., M. acerina, R. carotae, Sclerotium sclerotiorum Lib. or other fungi (Tronsmo 1989). The overall effect of the T. harzianum treatment on the different fungi was tested by two-way analysis of variance (ANOVA) using data of examination as blocks, and the frequency in each bag as single observations (n=3 for each treatment within each block).All fields were included in the ANOVA (each data treated as a separate block, without correction for differences between field trials). In some cases, however, ANOVA was sum separately for each field trial.

Results and discussion.

The treatment of carrots with a conidial suspension of T. harzianum P1 significantly reduced the amount of infected roots both from the M. acerina and the R.carotae infected fields (p=0.0002,

ANOVA through all trials). On average, the amount of marketable crop had increased by 47% after 6 months and 75% after 8 1/2 month in cold storage by the *Trichoderma* treatment.
The percentage of rot caused by *B. cinerea*, *M. acerina*, *R. carotae* and *S. sclerotiorum* on the control and *Trichoderma* treated carrots is shown in Fig 1. There was a significant reduction in the attack by *B. cinerea*, *R. carotae* and *S. sclerotiorum* in both the *R.carotae* and the *M.acerina* infected field.
This experiment indicates that biological control of storage rots on cold stored vegetables is possible. What can be done to increase the possibilities for commercial use of post harvest biological control? A strategy to obtain this goal is indicated in table 1. The first part of this strategy can be done in a research institute, but the last part has to be carried out in cooperation with a commercial company due to the costs involved.

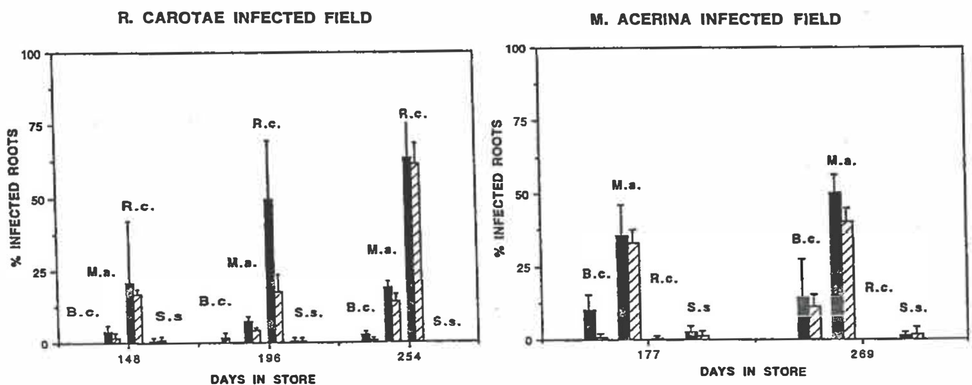

Fig 1 Percentage of carrots with visible disease symptoms of *Botrytis cinerea* (B.C.), *Mycocentrospora acerina* (M.a.), *Rhizoctonia carotae* (R.C.), and *Sclerotinia sclerotiorum* (S.s.) after different periods in cold storage. Control ▮ . Treated with *Trichoderma harzianum* P 1 before storage ▨ . Standard error of mean value is indicated by vertical lines.

Table 1.Strategy for development of biological control of post harvest pathogens.

I

1. Screen in the laboratory a large number of potential BCA for ability to grow at the recommended storage temperature on nutrition available on the surface or wounds of the produce.
2. Screen selected isolates under natural controlled conditions for: Growth rate on available nutrient. Effect of added nutrients. Antagonistic activity against target pathogens on the plant. Amount of antagonists needed for control.
3. Develop large scale production method for the BCA.

II

4. Large scale test under different realistic storage conditions and disease pressures.
5. Strain improvement.
6. Commercialization:
   Legislation of the BCA. Development of a large scale production method that gives a product with acceptable price and shelf life.Formulation of the BCA for the targeted crop.
   Marketing.

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RELATIONSHIPS BETWEEN THE MAIN PATHOGENIC FUNGI OCCURRING ON STORED CARROTS AND SOME SAPROPHYTIC FUNGI

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Summary

In the *in vitro* tests with 10 isolates belonging to five species of saprophytic fungi as potential antagonists of the main stored carrot pathogens *Sclerotinia sclerotiorum* and *Stemphylium radicinum* (=*Alternaria radicina*), only *Trichoderma viride* isolates exhibited antagonism to both pathogens.
Among the 15 mutants of *T. viride* derived from the isolate Td_5, resistant to TMTD and methyl thiophanate, 6 mutants (Td_D, Td_E, Td_N, Td_M, Td_A and Td_F) were antagonistic to both pathogens, the mutants Td_M and Td_N showing high antagonism to these.
It is estimated that they can be used as basic biological material for developing efficient bioproducts able to prevent harmful effects of stored carrot mycoflora.

Introduction

The main fungi causing stored carrots decay are *Sclerotinia sclerotiorum* (Lib.) de Bary, *Stemphylium radicinum* (Meier, Drechsler et Eddy) Neergaard (= *Alternaria radicina* Meier), *Botrytis cinerea* Pers., *Geotrichum candidum* Link et Pers., *Mycocentrospora acerina* (R. Hartig) Deighton (= *Centrospora acerina* (Hartig) Newhall, and various *Fusarium, Penicillium, Aspergillus, Mucor, Rhizopus* and *Rhizoctonia* species etc. The first three species being the most frequent and also the most harmful (Taşcă and Hulea, 1973; Taşcă, 1977; Hulea et al., 1982).
Commonly, their harmful effects are controlled by chemical means while other procedures, such as biological, are rarely used (Tronsmo, 1986, 1987, 1989; Wilson and Wisniewski, 1989; Whipps, 1991, 1992; Prokkola, 1992).
In order to reveal some biological means able to prevent and control the detrimental effects of mycoflora affecting stored carrots, we have tried to screen fungi antagonistic to the main pathogens of this flora.

Materials and methods

Two test-pathogenic fungus isolates of *Sclerotinia sclerotiorum* (Scl.m) and *Stemphylium radicinum* (St.) have been used as biological material, and also 10 isolates of saprophytic fungi belonging to 5 species and 15 *Trichoderma viride* Pers. ex S.F. Gray mutants, previously obtained (Şesan and Baicu, 1989; Şesan, 1990). In order to disclose the *in vitro* relationships between the two test-fungi, on one hand, and the isolates and saprophytic fungi mutants, on the other hand, the method of double cultures (Jouan et al., 1964) has been applied, these relationships being expressed by the values of the ratio (x) between internal (i) and the external radius (e) of the test-fungus (A) and of the antagonistic one (B), after the formula:

$$X = \frac{i_A}{i_B} \cdot \frac{e_B}{e_A}$$

Results and discussion

Among the 10 isolates tested (fig. 1) only *Trichoderma viride* isolates (Td_5, Td_{23}, Td_{49}, Td_{50}) demonstrated antagonism to *Sclerotinia sclerotiorum*, the x values ranging between 0.4 and 0.8. Isolates from *Gliocladium roseum* (Gl), *Trichothecium roseum* (Tt), *Verticillium tenerum* (Vt) and *Coniothyrium minitans* (Cm) were not antagonistic to *Stemphylium radicinum* (= *Alternaria radicinia*) than to *Sclerotinia sclerotiorum* (x = 0.7 - 0.9), except for the isolate Td_5, which strongly acted

antagonistic. *Verticillium tenerum* exhibited a slight antagonism (x = 0.9), whereas before of *Gliocladium roseum* (Gl), *Trichothecium roseum* (Tt) and *Coniothyrium minitans* (C.m.) did not show antagonism (x = 1.1. - 2.0). All *Trichoderma viride* mutants (Td_A .... Td_O), resistant to TMTD and methyl thiophanate, were in all cases more antagonistic than the isolate Td_5, which they derived from. A higher antagonism to *Sclerotinia sclerotiorum* was displayed, in decreasing order, by mutants Td_M, Td_N, Td_K, Td_C, Td_D, Td_E, Td_F, Td_I, Td_J, and Td_A (x = 0.2 - 0.4). The mutants Td_A, Td_D, Td_E, Td_F, Td_M and Td_N showed the highest antagonism to *Stemphylium radicinum* (x = 0.2 - 0.3). These six mutants also manifested high antagonism to the first pathogen of stored carrots (*Sclerotinia sclerotiorum*).

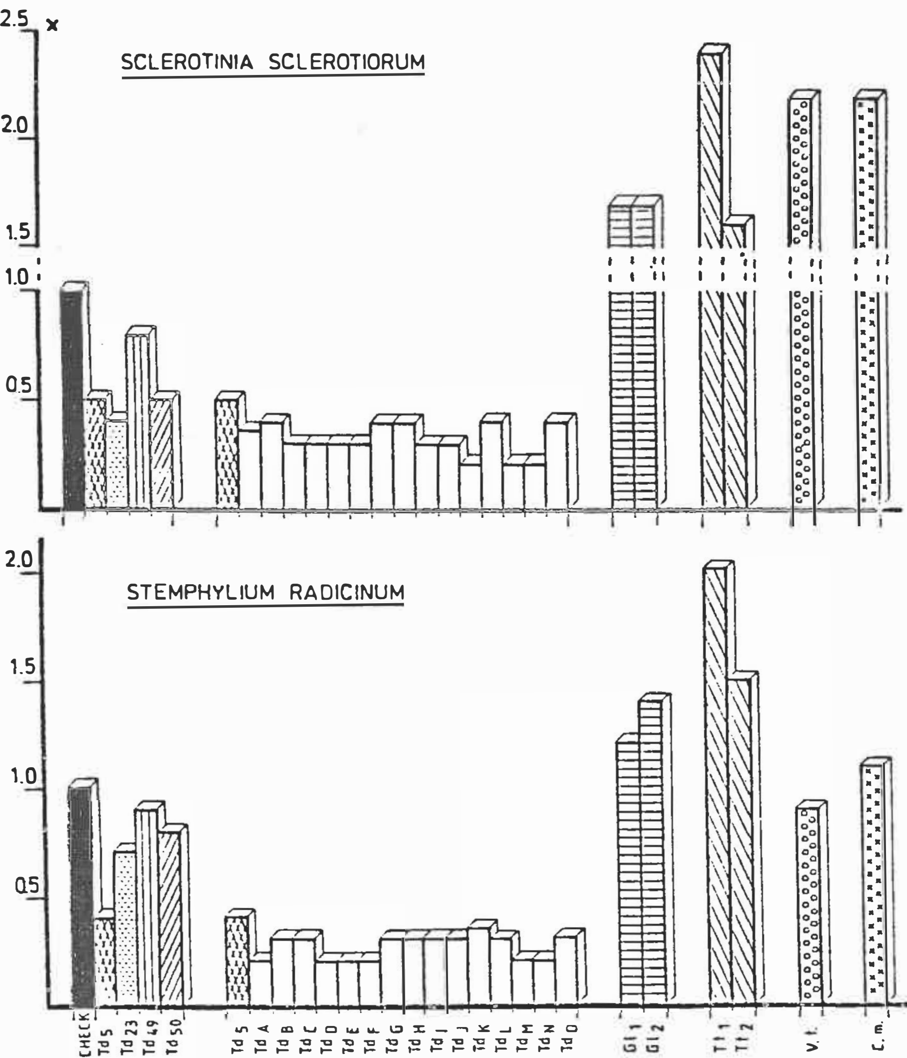

Figure 1 - "In vitro" antagonism of some saprophytic fungus isolates and Trichoderma viride mutants versus Sclerotinia sclerotiorum and Stemphylium radicinum occurred on stored carrots as expressed by x coefficient

From our results, as well as from literature, it resulted that only *Trichoderma* spp. isolates are highly antagonistic to both pathogens affecting carrots in storage. Consequently, further screening of isolates and mutants of microorganisms demonstrating high antagonistic activity would be necessary, with a view to their use as a starting point for prevention and control of harmful mycoflora of carrots during storage.

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BIOLOGICAL CONTROL OF POSTHARVEST BOTRYTIS CINEREA AND PENICILLIUM ON APPLES

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Abstract

Seven microbial strains were selected for their protective activity against *B. cinerea* and *Penicillium* sp. among 329 epiphytic microorganisms isolated from apple fruit surface. Ratio between inoculum concentration of antagonists and pathogens and time between application of the protective yeast and inoculation of the pathogens appeared to be important factors in controlling the level of protection. The application of 10^8 cfu/ml of the most efficient strains K (*Pichia anomala*) or O (*Candida sake*) is required to inhibit completely the lesions of *B. cinerea* inoculated with spore concentrations of 10^5 conidia/ml . Population of K and O in wounds increased at 25°C to reach a maximum after 12h of incubation and a relationship appeared between population size of the biocontrol agent and the level of protection. *In situ* spore germination of *B. cinerea* was markedly reduced on K or O-treated-wounded sites even when pathogen and yeast were applied simultaneously, thus suggesting that an other factor than inhibition of germination may interact in biocontrol effectiveness.

Introduction

The interest for alternative approaches to control diseases appeared since a few years. Progress has been made particularly in the biocontrol of postharvest diseases of fruits (JANISIEWICZ, 1991). The present work is dealing with two postharvest diseases of apples : gray mold caused by *Botrytis cinerea* Pers & Fr. and blue mold caused by *Penicillium* sp. LK. & Thom. Seven strains of yeasts were selected for their protective activity at 5 and 25°C among 329 epiphytic microorganisms (yeast and bacteria) isolated from apple fruit surface (cv Golden Delicious and Jonagold) when applied to wounded apples inoculated 24h later with 50 μl of 10^6 spores/ml of both pathogens (unpublished results).

A more precise screening of antagonistic strains and identification of the major parameters controlling the level of protection constitued a prerequisite in order to approach the mechanism of action of protective yeasts. The second objective of the present study was to determine the effect of biocontrol agents (BCA) on conidial germination of *B. cinerea* with a *in situ* method.

Material and methods

Surface sterilised-Golden Delicious apples were inoculated by depositing 50 μl of *B. cinerea* or *Penicillium* sp. spore

suspension on wound sites (2 wounds of 6 mm diameter and 3 mm deep at the equator per apple) previously treated with 50 μl of yeast suspension (in sterile 0.1% pepton water). Inoculated fruits were incubated on wet filter paper in closed plastic boxes at 25°c and symptom intensity (as measured by the diameter of the lesion) was evaluated 5 days after the inoculation
The protective effect of BCA was studied in relation to the time between antagonist application and pathogen inoculation as also the concentration of yeast cell and pathogen spore suspension.

The ability of the antagonist to colonize the inoculation site was investigated by inoculating wounded fruits (one wound 20 mm in diameter, 1 mm deep per appel) with 50 μl of antagonist suspension (10^8 cfu/ml). After increasing incubation times (0 to 72h) wound sites were blended and dilution-plated in triplicate onto PDA for assay of colony formings units (cfu). Parallel with population studies, the level of protective activity against *B. cinerea* was measured with the same experimental protocol.

In order to determine the effect of BCA on conidial germination of *B. cinerea* on apple, inoculum of the pathogen was applied on a transparent menbrane filter (0.2 μm pore diameter) put in the antagonist-pretreated wound sites. 24h after inoculation the percentage of germination of *B. cinerea* was determined under light microscopy.

All experiments were repeated at least once. Factorial analyses of variance for the effect of incubation time and inoculum concentration were performed with models procedure of SAS.

Results and discussion

1. Effect of duration between treatment of antagonistic yeast and inoculation of pathogen and the effect of the yeast cell and pathogen spore concentration on the biocontrol efficacy.

Protective activity of yeasts against *B. cinerea* and *Penicillium* sp. increased with the incubation time between the application of the antagonist and inoculation of pathogen (table 1). The most efficient strains (K and O) reduced significantly the diameter of decay lesion even when inoculation of the pathogen and application of the yeast were performed simultaneously. Application of strains K (*Pichia anomala*) and O (*Candida sake*) gave rise to 90-100% of inhibition of lesion of both pathogen inoculated 48h later (table 1).
There was a quantitative relationship between spore concentration of the pathogens and the amount of antagonist needed for disease control (tables 2). The application of 10^8 cfu/ml of strain K is required to inhibit completely the lesions of *B. cinerea* caused by spore concentration of 10^6 conidia/ml whereas 10^8 cfu/ml of strain O protected fruits inoculated with 10^5 spores of *B. cinerea*/ml.

Table 1: Lesion development (mm) on wounded Golden Delicious apples treated with 50 µl of antagonistic yeast suspension (about 107 CFU/ml) and inoculated with 50 µl of pathogen suspension (106 spores/ml) after different incubation times of the antagonist

	Botrytis cinerea				*Penicillium* sp.			
Incubation time	0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
2.13[b]	21,6[a]	9,4[d]	6,9[d]	0,5	20,2	15,6[d]	6,9[d]	5,4[d]
1.58	24,9	12,2[d]	11,2[d]	9,1	16,4[d]	15,6[d]	14,2	9,0
9C5	24,7	19,5	7,0[d]	3,1[d]	20,2	22,6	19,1	10,4
5F2	27,0	13,9	7,1[d]	19,7[d]	18,5	16,7[d]	15,5	4,1[d]
K	10,7[d]	8,6[d]	0,0[d]	0,1[d]	10,6[d]	12,2[d]	3,7[d]	0,0[d]
O	15,2[d]	5,7[d]	4,7[d]	3,0[d]	19,2	18,1	3,1[d]	2,0[d]
9A4	22,0	19,5	14,1[d]	5,4[d]	25,4	22,6	19,2	14,2
control[c]	33,8	25,1	31,9	29,4	23,6	23,4	22,6	19,0

a: Data represent average lesion diameter (mm) measured 5 days after pathogen inoculation.
b: Antagonistic strains.
c: Apples inoculated only with the pathogen.
d: Mean of the antagonist-treated apples is significantly different ($p = 0,001$) to the control mean according to Dunnett's procedure.
Remark: Data shown for 1 of the 2 trials.

2. Effect of population densities of strains K and O on conidial germination and on level of protective activity against *B. cinerea*.

Population of K and O in wounds increased at 25°C to reach a maximum after 12h of incubation (fig. 1). 72h after their application, the population level was approximately 1 log unit over the initial population level. A close relationship appeared between population of the biocontrol agent and the level of protection. The percentages of reduction of *B. cinerea* inoculated 0, 4, 8, 12, 24 and 48h after the antagonist application were respectively 21.4, 27.2, 38.7, 72.5, 97.3 and 98.1 % on K-treated fruits and 30.9, 35.3, 44.8, 81.8, 98.3 and 100.0 on O-treated fruit.
Spore germination of *B. cinerea* was reduced on K or O-treated-wound sites. After 0, 4, 8, 12, 24 h of incubation of yeasts, spore germinations of *B. cinerea* on membrane filters were 14, 19, 14, 10, 6 % respectively on K-treated apple and 20, 15, 15, 12 and 9 % in case of O application. The conidial germination of control (untreated fruit), after the same incubation times as above described were 58, 73, 83, 60, 45 %.

These overall results suggest that protection effect of yeast was closely related to the colonization. *In situ* spore germination of B. cinerea was markedly inhibited even when pathogen and yeast were applied simultaneously with no subsequent protection, thus suggesting that other factor(s) than inhibition of germination may also interact in biocontrol effectiveness. Additional studies are needed to confirm the *in situ* spore germination and to determine if antifungal metabolites are produced by K or O and/or apple cells in the wound site.

TABLE 2: Lesion development (mm) on wounded Golden Delicious apples inoculated with various spores concentrations of *B. cinerea* or *Penicillium* sp. 24 h after treatment of different concentrations of *Pichia anomala* or *Candida sake*.

	B. cinerea spores concentration (spores/ml)				*Penicillium* sp. spores concentration (spores/ml)			
Yeast concentration (CFU/ml)	10^7	10^6	10^5	10^4	10^7	10^6	10^5	10^4
Pichia anomala								
10^8	3,5[ad]	0,0[d]	0,0[d]	0,0[d]	4,6[d]	0,0[d]	1,1[d]	0,0[d]
10^7	4,7[d]	0,7[d]	1,4[d]	1,6[d]	9,4[d]	2,6[d]	0,0[d]	3,7[d]
10^6	17,0	5,2[d]	10,2[d]	0,0[d]	17,2	18,0	14,1	20,2
10^5	24,4	11,2[d]	5,9[d]	5,0[d]	18,1	12,9[d]	15,4	4,9[d]
control[b]	24,5	28,1	25,0	22,1	21,0	21,4	21,6	19,1
Candida sake								
10^8	9,5[ad]	2,9[d]	0,0[d]	0,0[d]	4,7[d]	6,2[d]	8,5[d]	4,6[d]
10^7	14,2	2,6[d]	3,4[d]	0,0[d]	15,5	4,0[d]	2,1[d]	0,0[d]
10^6	17,1	18,6[d]	9,1[d]	6,7[d]	11,0	15,2	10,1[d]	4,7[d]
10^5	13,2	7,9[d]	2,6[d]	0,9[d]	14,9	16,1	8,4[d]	9,2[d]
control[b]	24,5	28,1	25,6	22,1	21,0	21,4	21,6	19,1

a: Data represent average lesion diameter (mm) measured 5 days after pathogen inoculation.
b: Apples inoculated only with pathogen.
c: Mean of the antagonist-treated apples is significantly different to the control mean according to Dunnett's procedures (P = 0,001)

Remark: Data shown for 1 of the 2 trials

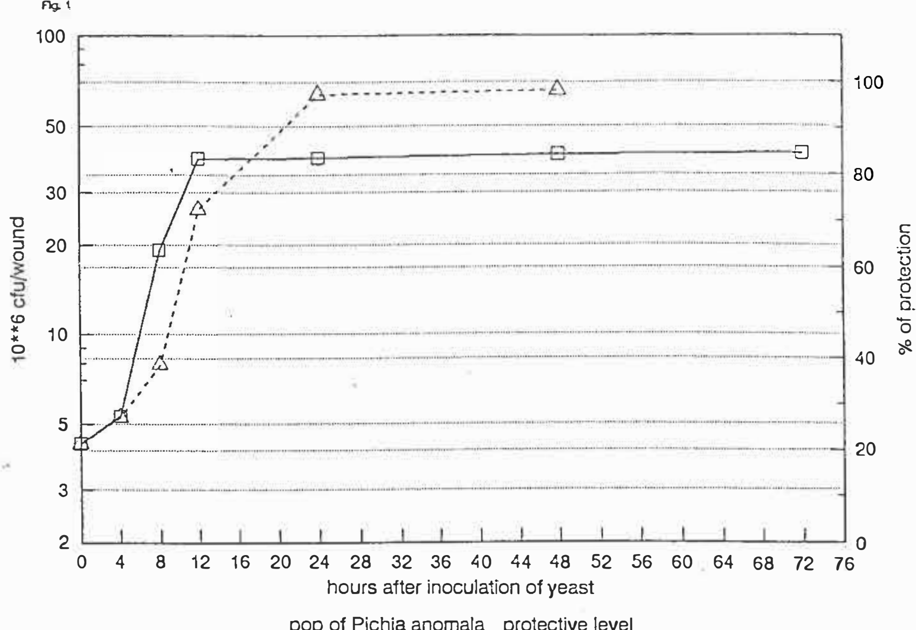

Fig. 1 : Effect of population densities of *Pichia sake* on level of protective activity against *B. cinerea*. Data for population densities represent mean number of colonies from three trials (one wound site/trial). Each wound was triplicate-plated. Data for protective level represent the mean % of protection (as compared to the control which was not treated with yeast before inoculation of *B. cinerea*) from 2 trials (6 wounds/trial). Bars represent standard error of the mean.

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This work was financially supported by the EEC (project CAMAR n°80012 CT91-0106)

ENHANCING BIOCONTROL OF POSTHARVEST DISEASES OF APPLE WITH NITROGENOUS COMPOUNDS.

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Summary
Biocontrol of postharvest diseases of apple is emerging as a promising alternative to fungicidal treatment. However, implementation of this method of control will largely depend on its reliability and economics. Applications of higher concentrations of the antagonists to fruit increase reliability of control but reduce the economics. Thus, a method was developed to enhance biocontrol of Penicilium expansum by adding selected nutrients which stimulate the antagonist on fruit and eliminate the need to apply higher concentrations of the antagonist.

Introduction

In the past decade great progress has been made in biocontrol of postharvest diseases of pome fruit. Private industry has begun exploring commercial potential of some biocontrol agents. Two factors, reliability and economics, will have great impact on the feasibility of using this method of control. A number of factors influence the effectiveness of biocontrol agents on fruit, they include: concentration of a pathogen propagules, fruit maturity, cultivar, wound type, postharvest treatments, and storage conditions. Higher concentrations of the antagonist are required to achieve acceptable control on some cultivars, on more mature fruit, and on fruit with severe wounds. However, the use of higher concentrations of antagonists increases cost. The great advantage of a postharvest biocontrol system is that the physical and chemical environment can be manipulated to enhance the effectiveness of the antagonists. The objective of this work was to enhance biocontrol of Penicillium expansum on mature apple by stimulating population of the antagonist Pseudomonas syringae (L-59-66) with nutrients.

Methods

1. Effect of nutrients on antagonist and pathogen.

Several carbohydrates and nitrogenous compounds were tested for their effect on growth of the antagonist, Pseudomonas syringae (L-59-66), and on germination, germ tube and radial growth of Penicillium expansum. The tests for utilization of these compounds by the antagonist were conducted in microtiter plates containing basic minimum salt medium with single nitrogen or carbon source, and tetrazolium dye. The antagonist suspension was applied to the plates and incubated for 24 hr at 24 C. Then absorption by the wells was determined with a plate reader at 590 nm.

Higher levels of absorption indicated greater utilization of the compound. Tests for conidia germination, germ tube, and mycelial growth were conducted in petri plates (30 mm) with 1.2% Phytagel medium containing one of the carbohydrate or nitrogenous compounds. Germination and germ tube growth tests were conducted by spreading 100 ul of a conidia suspension over the medium surface. Plates were incubated at 20 C for 24 h and conidial germination and germ tube growth were determined microscopically. To measure colony growth, plates were inoculated in the center with a 3-mm diameter and 1-mm thick plaque removed from the margin of a colony actively growing on PDA medium. Plates were incubated for 168 h at 20 C, and the diameter of the colony was measured.

2. Fruit tests.

Compounds that stimulated the antagonist growth most but had little effect on the pathogen germination or growth were tested for enhancing biocontrol of P. expansum on apples in laboratory tests. Wounded apples were treated with solutions containing a mixture of various concentrations of the antagonist and nutrients, and then inoculated with $1x10^4$ conidia/ml of the pathogen. Concentrations of carbohydrates ranged from 0.05 to 12.8 mg/ml, and nitrogenous compounds from 5 to 80 mM. The fruit were incubated in boxes for 7 days at 22 C and the diameter of the lesion was measured.

Based on laboratory tests, L-proline and L-asparagine were selected to enhance biocontrol of P. expansum in larger scale, semi-commercial tests. The antagonist-pathogen-nutrient mixture was sprayed on fruit on the sorting line. Concentrations were 5.4×10^8 cfu/ml, 1×10^4 conidia/ml and 80 mM for the antagonist, pathogen and amino acids, respectively. Fruit were put into polyethylene liners in one-bushel boxes and stored at 1 C for up to nine months. At three-month intervals fruit were removed from storage for rot evaluation.

3. Recovery of the antagonist.

Fruit designated for recovery tests were treated with the antagonist similarly to biocontrol tests. In a laboratory test, antagonist populations were determined 2, 4, 8, and 16 days after application. In the semi-commercial trial, recovery of the antagonist was conducted when fruit rot was evaluated.

Results and Discussion

Ranking of the compounds according to their effect on germination and germ tube growth of conidia of P. expansum were similar; thus references are made only to conidia germination. Germination of the conidia was not stimulated by 10 carbohydrates, and in the case of another 10, less than 2% germinated.

Fourteen out of 36 carbohydrates and seven out of 23 nitrogenous compounds were strongly utilized by the antagonist. All nitrogenous compounds affected germination and radial growth of P. expansum; but only L-asparagine and L-proline significantly enhanced biocontrol on Golden Delicious apple (Fig. 1). In laboratory tests, lesion size decreased as concentration of the amino acids increased. At the highest concentration of the antagonist lesions were reduced from 8 to 0 mm and from 17 to 4 mm for L-asparagine and L-proline, respectively. When the antagonist was applied to fruit on the sorting line, each amino acid significantly enhanced biocontrol (Table 1). Less than 2 % of the wounds were infected after six months in storage.

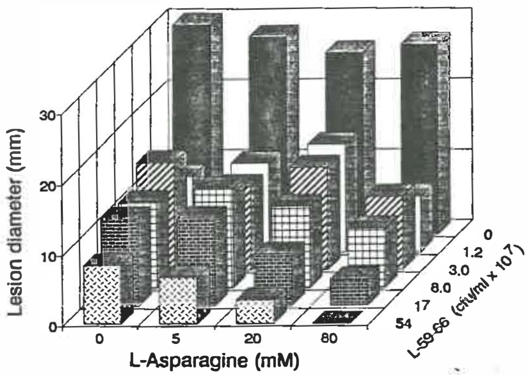

Fig.1. Effect of L-asparagine and antagonist (Pseudomonas syringae strain L-59-66) concentrations on lesion diameter of blue mold on Golden Delicious apple, after seven days at 22C in laboratory tests.

Both amino acids strongly stimulated populations of the antagonist on fruit in laboratory and large scale tests. In the laboratory tests P. syringae populations increased by approximately two orders of magnitude for

Table 1. Percentage of wounds infected on wounded Golden Delicious apple treated on sorting line with solutions containing various combinations of conidia of Penicillium expansum, antagonist Pseudomonas syringae (L-59-66), and amino acids L-proline (Pro) and L-asparagine (Asn) and stored for various periods of time at 1 C.

	Infected wounds (%)		
Treatment	3 months	6 months	9 months
Control	63	72	75
L-59-66	5	13	24
Control Pro	51	78	71
L-59-66 + Pro	1	1	6
Control Asn	48	72	83
L-59-66 + Asn	0	1	8

L-asparagine and L-proline, compared to one order of magnitude for control during the first two days (Fig. 2). Similar changes in population sizes were observed on fruit from large scale tests after three months of storage at 1 C; however, the differences were smaller after six months of storage and almost completely disappeared after nine months when populations began to decline.
The amino acids that enhanced biocontrol also strongly stimulated conidia germination but had much less effect on radial growth of P. expansum. Thus, in this biocontrol system, inhibition of conidial germination may not be as important as inhibition of subsequent stages of pathogen development. Apples

are a rich source of carbohydrates but a poor source of nitrogen; thus it is not surprising that amino acids stimulated the antagonist and enhanced biocontrol.

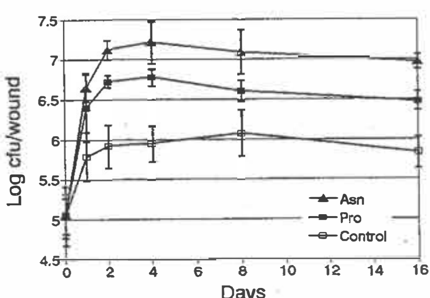

Fig.2. Effect of L-asparagine (Asn) and L-proline (Pro), both at 80 mM concentrations, on population dynamics of the antagonist (Pseudomonas syringae strain L-59-66) in wounds of Golden Delicious apple.

In other biocontrol system on the phylloplane, with the nectrotrophic pathogen Botrytis cinerea, amino acids become limiting nutrients before carbohydrates do (1). Rapid stimulation of the antagonist population by both amino acids was probably the decisive factor in enhancing biocontrol, since the outcome of biocontrol to a large extent is determined during the first 48 h after application of the antagonist at 22 C.
Our research has demonstrated that nutrients can enhance biocontrol of postharvest diseases of ripe fruit without further increasing the concentration of the antagonist applied. The probability of successfully finding stimulatory compounds can be increased by a rational selection of compounds in vitro tests. However, nutrients used to enhance biocontrol of one pathogen must not stimulate other pathogens. In our study, both nutrients also enhanced biocontrol of B. cinerea, but the effects were stronger with P. expansum (2). This approach may be useful in enhancing biocontrol of postharvest diseases of fruits and vegetables in other pathosystems.

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BIOLOGICAL CONTROL OF THE POST-HARVEST PATHOGENS *PEZICULA MALICORTICIS, NECTRIA GALLIGENA, BOTRYTIS CINEREA, MONILINIA FRUCTIGENA* and *PENICILLIUM EXPANSUM.*

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Summary

During the seasons of 1988-1990, over 2270 isolates of microorganisms were collected from apple trees and tested *in vitro* and *in vivo* against the post-harvest pathogens *Pezicula malicorticis, Nectria galligena, Botrytis cinerea, Monilinia fructigena* and *Penicillium expansum.* Many isolates *in vitro* inhibited spore germination or mycelial growth of one of the pathogens. Similar results were obtained *in vivo* with wounded apples kept at 4°C at high humdity. No microorganism inhibited all five pathogens at a similar level. Therefore, we tested combinations of isolates. Very often, a 5:1 ratio of antagonist to pathogen was enough to obtain good control with wounded apples. We also performed experiments in the field. Apple trees were sprayed two times, three and one week before harvest respectively. Results with some antagonists were comparable to those after fungicide treatment. However, antagonistic fungi were very sensitive to fungicide sprays.

Introduction

Several pathogens contribute to post-harvest losses of apples. *Botrytis cinerea, Penicillium expansum* and *Monilinia fructigena* are the major post-harvest pathogens. In addition, *Pezicula malicorticis* and *Nectria galligena* may infect up to 30 % of stored apples (Kennel, 1988) through wounds or lenticells. Currently, fungicide treatments are performed routinely before harvest. As an alternative to this reliance on chemical control, antagonistic microorganisms have been used to prevent the infection of apples by blue mold (Janisiewicz *et al.*, 1992), gray mold (Roberts, 1990), *Pezicula* and *Nectria* spp.(Schiewe and Mendgen, 1992). We isolated microorganisms which occur throughout the year from fruits and leaves and tested them for activity against all five pathogens (Falconi and Mendgen, 1993). Effective mixtures of antagonists were applied in a field experiment.

Results

At two week intervals, bacterial and fungal isolates were randomly collected during 1988, 1989 and 1990 from apple leaves and fruits. These microorganisms were tested for their ability to inhibit spore germination, mycelial growth on agar or to inhibit rot development on apples wounded (3 x 3 mm) with a cork borer. Table 1 shows the influence of some *Aureobasidium pullulans* and *Trichoderma polysporum* isolates on *B. cinerea* (B.c.), *M. fructigena* (M.f.) or *P. expansum*

(P.e.). Results from the other two pathogens (N. Galligena and P. malicorticis) have been published recently (Schiewe and Mendgen, 1992). In short, we found no antagonist which was equally effective to inhibit spore germination, mycelial growth or rot development. Also, no antagonist inhibited the five post-harvest pathogens at a similar level. After the application of the antagonists into apple wounds, lesion size was largely dependent on the ratio pathogen to antagonist within the wound. Fig. 1 shows the influence of an isolate from Bacillus subtilis on lesion development produced by B. cinerea and M. fructigena after two weeks storage at 4°C. It is evident that a pathogen to antagonist ratio of 1:10 was required in this case. Results with other bacteria and fungi also indicated that the effective ratios were largely independent on absolute numbers of bacteria or fungi respectively. With some fungi, a ratio 1:1 or 1:5 pathogen to antagonist was sufficient to inhibit fruit decay. In a field experiment performed in 1991, a mixture of antagonists (KN-1503; KN-7574; I-681) with 10^6 propagules/ml was sprayed two times (16.9.91 and 1.10.91). Control trees were sprayed either with water or with fungicides (0,15% Euparen). The fruits were harvested by the farmers and stored under cool room conditions (+5°C ±2) as usual.
In March, fruit decay was evaluated (Table 2). Results after treatment with the antagonists were similar to results obtained after application of the fungicide. In a different experiment, antagonists were applied first and fungicides subsequently. These preliminary experiments indicated that antagonistic fungi are very sensitive to fungicide treatment (results not shown).

Table 1 Inhibition of spore germination, mycelial growth and the development of apple lesions by B.cinerea (B.c.), M.fructigena (M.f.), and P.expansum (P.e.)

Isolate		INHIBITION (%)								
		Spore germination			Mycelial growth			Apple lesions		
		B. c.	M. f.	P. e.	B. c.	M. f.	P. e.	B. c.	M. f.	P. e.
Aureobasidium pullulans	KC1503	60	20	45	8	4	20	96	95	75
	KC1506	52	19	44	4	12	22	70	90	78
	KC1507	56	18	40	3	18	25	87	92	64
	KC1512	58	13	44	5	10	21	80	50	60
Trichoderma polysporum	KC1014	85	90	90	95	98	99	93	80	87
	KC1218	84	89	88	92	97	98	90	75	80
	KC1393	84	83	80	90	94	98	89	60	78
	KC1412	83	86	90	85	92	80	85	72	65
	KC1553	80	90	94	80	90	74	70	68	42
	KC1684	98	90	87	85	90	96	28	12	6
	KC1714	92	98	90	80	75	92	14	38	10

Fig.1 Influence of different concentrations of antagonist B.subtilis, isolate KN 67, on lesion development produced by different concentrations of B.cinerea and M.fructigena.

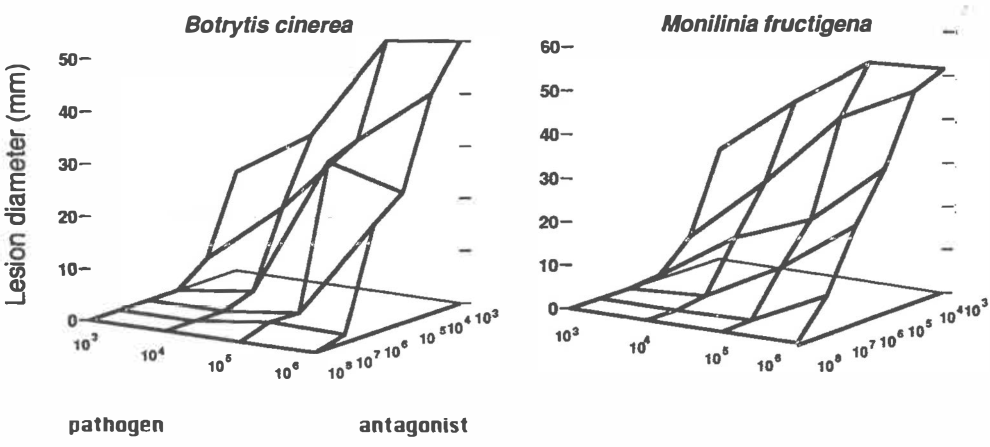

Table 2 Fruit decay (%) after three different treatments in the field experiment 1991/1992

	Water-control	Fungicide	Antagonists
Pezicula spp.	63	5,6	24
Nectria galligena	17	12	8,1
Botryis cinerea	2,5	2,5	0,4
Monilinia fructigena	3,8	1,3	0,6
Penicillium expansum	0,3	0,2	0,1
Other	13	23	9,4
Total	100	44	43

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PILOT TESTING OF PICHIA GUILLIERMONDII - A BIOCONTROL AGENT OF POSTHARVEST DISEASES OF CITRUS FRUIT.

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Summary

The efficacy of the yeast Pichia guilliermondii (US-7) in controlling postharvest decay of citrus fruit was evaluated in small-scale and pilot tests in commercial packinghouses. Scale-up fermentation of US-7 has resulted in an efficient growth of the yeast on cheap industrial waste materials while maintaining its antagonistic activity, as tested in vitro, against spore germination of Penicillium digitatum. In small-scale experiments with injured and non-injured fruit dipped in the yeast cell suspension, the development of decay in several citrus cultivars was effectively inhibited. The results indicated that the yeast was compatible with commonly used waxes containing a low concentration of a chemical fungicide. In packinghouse tests, combining the yeast with 200 ppm TBZ resulted in a reduction of decay to a level equal to that of the commercial treatment and decreased variability in the performance of the biocontrol treatment. It was also found that the efficacy of US-7 could be maintained under packinghouse conditions at a cell concentration as low as 10^7 to 10^8 cells/ml.

Introduction

Public and scientific concern about the presence of synthetic pesticides in our food supply and in the environment has increased in recent years. In addition, development of resistant strains of pathogens to postharvest fungicides (Eckert, 1990) has contributed to diminishing our ability to control postharvest diseases of fruits and vegetables. As a result of these mounting concerns, considerable efforts have been made in recent years to evaluate the potential of using antagonistic microorganisms as an alternative to the use of synthetic chemicals for the control of postharvest diseases of fruits and vegetables (Wilson and Wisniewski, 1989). Several naturally occurring antagonistic microorganisms were found effective in the control of a number of different postharvest diseases of various fruits and vegetables (Wisniewski and Wilson, 1992).

In previous studies, a yeast, Pichia guilliermondii Wickerham (US-7), isolated from the surface of lemon fruit (Wilson and Chalutz, 1989), exhibited antagonistic activity against a range of postharvest pathogens of fruits and vegetables (Chalutz and Wilson, 1990; Droby et al., 1991). We report here the results of large-scale experiments and pilot tests of this yeast antagonist conducted in commercial citrus packinghouse lines aimed at evaluating its commercial potential for the control of postharvest decay of citrus fruit.

Materials and Methods

Scale-up fermentation and batch efficacy tests

The scale-up and optimization of the fermentation process was performed in a 15 l bench-top fermentor (Microgen, New-Brunswick). The growth medium for initial production of US-7 was NYDB. Fermentation was also performed on media composed of industrial waste materials such as cotton-seed meal or corn-steep liquor or partially digested peptones.
The biocontrol efficacy of yeast preparations was tested in culture on spore germination of *P. digitatum* in a synthetic medium and also on grapefruits, as previously described (Wilson and Chalutz, 1989).

Small-scale experiments

Ability of US-7 to reduce decay of naturally-infected fruit was tested on non-wounded and wounded citrus fruit. In non-wounded fruit, the varieties Temple and Topaz, hybrids of Tangors and Tangelos (*Citrus reticulata*) and Shamouti (*C. sinesis*) were used 24 h after harvest. Fruit was dipped momentarily in a cell suspension of US-7 (10^8 cell/ml), allowed to dry for 1 h, and then packed in commercial cartons and stored at 17 C. Following two weeks of storage, the development of decay was determined. For wounded fruit tests, Marsh seedless grapefruits (*C. paradisi*) were wounded with a dissecting needle to make wounds, 3 mm in diameter and 2 mm deep, at three locations equally separated from each other on the peel of the fruit. After wounding, fruits were dipped in the yeast cell suspensions (10^8 cell/ml) with or without the addition of 50 ppm thiabendazole (TBZ) (2-4-thiazolyl benzimidazole; Merck S & D). Following the treatment, fruit were dried for 2 h and then dipped in a commercial polyethylene-based wax, air dried and packed in commercial cartons. The packed fruit was stored at 11 C and percent infection of wounded sites was determined at weekly intervals for 10 weeks, followed by one additional week of shelf-life at 17 C.

Packinghouse tests

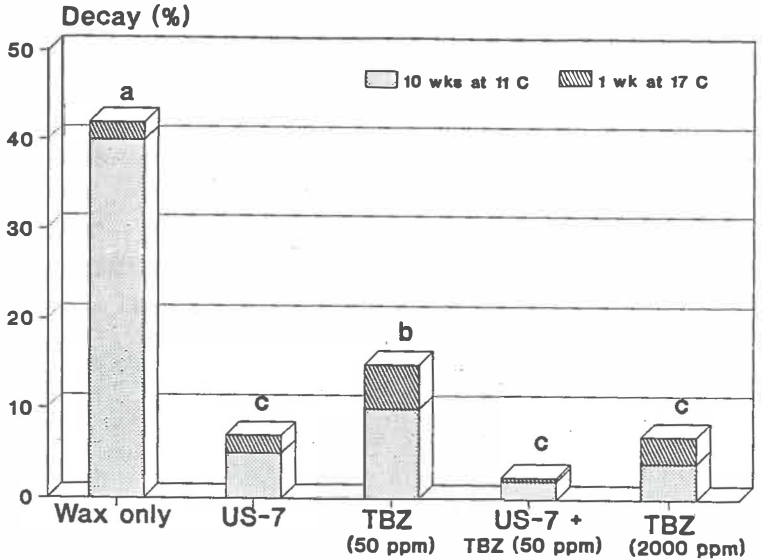
The yeast preparations used in all packinghouse tests were cultured in an 11-L fermentor on NYDB. Cells were harvested, washed and resuspended in water prior to use. Two methods of yeast cell application were assessed: 1) spraying the antagonist cells through standard nozzles and 2) drenching of the fruit on elevating rollers. Both methods were found satisfactory. The treatments were applied on commercially handled citrus varieties and were integrated with the routine of the packinghouse operation, except for the chemical fungicide application, which was replaced by treatments utilizing a cell suspension of the antagonist. Following the treatment, the packed fruit was stored at 17 C (85% RH) and the development of decay was determined after two weeks. Packinghouse tests were conducted during two successive seasons (1989/1990 and 1990/1991).

Results and Discussion

The results of the scale-up fermentation experiments indicated that both the rate of growth and the antagonistic activity of US-7 was maintained following fermentation on cheap waste materials, compared with fermentation in NYDB.
As reported previously for grapefruit, (Chalutz and Wilson, 1990), non-injured Topaz, Temple and Shamouti fruits dipped in the antagonist cell suspension, exhibited a reduced rate of natural infections compared with control fruit dipped in water.

Tests of the compatibility of US-7 with a low concentration of the fungicide TBZ and with a commercial citrus wax during prolong storage of grapefruit at 11 C indicated high efficacy of the biocontrol treatments (Fig. 1). The results suggested that the antagonist was compatible with the fungicide and with the wax treatment, and that it was highly effective in protecting surface wounds throughout the storage and shelf-life periods. A high cell count of the yeast was maintained on the fruit.

Figure 1 Inhibition of natural infections of *P. digitatum* by US-7 in artificially-wounded grapefruit during prolonged storage.



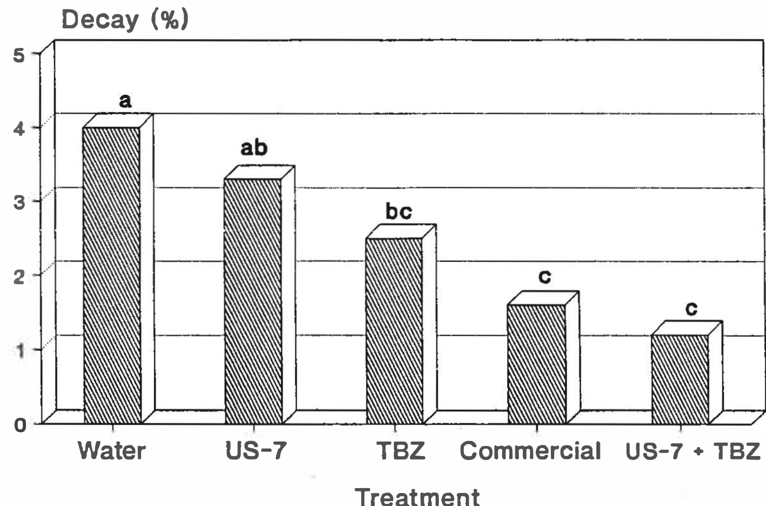
Maintaining high efficacy of the antagonist under cold storage conditions may be due to its ability to grow faster and survive under low temperatures compared with the pathogen.

Under packinghouse conditions, efficacy of the antagonists in controlling decay was generally maintained when a low concentration of TBZ was added to the antagonist cell suspension (Fig. 2).

Variability in the performance of the biocontrol activity was expected and could result from several factors: the initial quality of the fruit used in each test; the inoculum density; the method of application of the biocontrol agent; the sequence of the packing line; the susceptibility of the fruit to decay and the time elapsing between picking and treatment. To overcome this variability, a low concentration of TBZ (200 ppm, 1/10 to 1/20 of the commercially recommended concentration) was added to the US-7 preparations in most subsequent packinghouse tests.

Several sets of fruit obtained from different orchards were used in a series of packinghouse tests aimed at comparing the efficacy of US-7 (containing 200 ppm TBZ) with the standard commercial treatment. In three different sets of fruit, the incidence of decay which developed in the US-7-treated fruit was similar to the commercially treated fruit. In each test, the difference between the two treatments of the same sets was not statistically significant (at 95% level) in spite of the variability in the incidence of decay among sets, which originated from different orchards.

Figure 2 Efficacy of US-7 supplemented with 200 ppm TBZ against postharvest decay of citrus fruit (cv. Topaz) treated under packinghouse conditions.



The predominant decay pathogens observed in all packinghouse tests were P. digitatum and P. italicum, the causal agents of the green and blue molds, respectively. The incidence of sour rot increased only in tests conducted late in the growing season.

The results indicate the feasibility of large-scale production and application of P. guilliermondii (US-7) as a biological control agent of postharvest diseases of citrus fruit. They suggest that, in order to achieve high efficacy comparable with the standard commercial treatment and low variability, US-7 activity need to be enhanced by the addition of low concentrations of TBZ (200 ppm). Other efficacy enhancing methods are currently being explored as an alternative for the use of TBZ.

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INHIBITORY AND PHYSIOLOGICAL PROPERTIES OF 12 YEASTS AND BACTERIA ANTAGONISTIC TO BOTRYTIS CINEREA ON STRAWBERRY FRUITS

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INTRODUCTION

Grey mould, caused by B. cinerea is one of the most important diseases on strawberries. The pathogen attacks essentially flowers and fruits ; occasionally, other parts of the plant may be infected. Latent infections can occur on flowers, resulting in rot after harvest. This phenomenon can also occur on leaves, so that senescent leaves form a nutritive base and a source of contamination for harvested fruits (Sutton, 1990). When environmental conditions are favorable (northern oceanic climates), losses can be very important, even if chemical treatments are applied.
Biological control of diseases is an emerging area of research, fostered by the observation of an increasing number of fungicide-tolerant strains of pathogens and a public pressure to reduce pesticides in the food chain. Trichoderma applied to stawberry plants in the field partially controlled grey mold on strawberry fruits after harvest (Tronsmo and Dennis, 1977).
Expectation of total control of B. cinerea during the period of strawberry production using BCA, may seem unreasonable, but their use may reduce latent infections and post-harvest external contaminations of fruits. Additionally stawberries are characterized by a very limited post-harvest life because of their fragility and physiological evolution. Biological control should start in fields, and continues after harvest to protect strawberries and prolong their shelf life.

The present work was initiated to develop biological control of post-harvest diseases of stawberries, in the framework of a project funded by the European Community. At this time, work is focussed on the most important disease : rot caused by B. cinerea. To realise this first project we have isolated antagonists from the epiphytic microflora of fruits during storage. Twenty four potential antagonists were first isolated. We supposed because of their origin that they could resist and adapt easily to the drastic conditions of the strawberry storage. Finally, 12 strains (8 yeasts and 4 bacteria) were selected for their ability to inhibit B. cinerea on wounded fruit, when inoculated at a concentration of 10^6 CFU/ml ($P<0.05$, Tukey's multiple range test). This paper describe microbial characteristics, involved in the efficiency of the 12 antagonists.

METHODS AND RESULTS

We investigated first their activity on wounded fruit. A drop of water suspension of the agent (10^5 to 10^4 CFU/ml) was applied on the wound, challenged 3 hours later by a drop of spore suspension of B. cinerea. Fruits at the orange coloured stage were so treated and incubated 4 days at 16°C.

Secondly, their inhibitory effect was detected in vitro, in confrontation test on Potato Dextrose Agar (PDA) and Malt agar. The colonies were incubated at 19°C during 7 days.
Thirdly, the survival of the antagonists was tested on leaf disks under high relative humidity at room temperature and light. A drop of suspension containing almost 10^4 to 10^5 CFU/ml was applied on each disks. Three disks per strain were analysed at 3, 7, 14, and 21 days after inoculation. For each strain, the colonie type was identified by comparing to colony type from a freshly inoculated disk and verified by its absence on non-inoculated disks. Yeasts were counted on acidified Yeast Malt extract agar and bacteria on Yeast Peptone Glucose Agar supplemented with actidione (80mg/l).

Inhibitory activity at low concentration on wounded fruit
When the inoculum concentration is less than 10^6 CFU/ml, only bacteria had significant effect (Table 1). Among bacteria, one strain still possessed high inhibitory power at a concentration of 10^4 CFU/ml (which corresponds to 150 CFU per wound). In contrast, yeasts needed inoculum levels of 10^6 CFU/ml to be efficient and no significant difference appeared between them.

Table 1. ANTAGONIST ACTIVITY OF VARIOUS STRAINS ON WOUNDED FRUITS, AT LOW CONCENTRATIONS

Strains[1]	Lesion diameter (mm) — Concentration of antagonist	
	10^5CFU/ml	10^4CFU/ml
5B4*(B)*	8.05 a[2]	8.25 a
10B1'*(B)*	9.65 ab	12.20 b
5B4'*(B)*	11.50 cd	12.25 b
10B6*(B)*	11.30 bc	12.70 bc
10cL4*(Y)*	13.30 defg	13.25 bcd
5L3*(Y)*	12.80 cdefg	13.25 bcd
10cL3'*(Y)*	12.05 cde	13.70 bcd
10L2*(Y)*	12.70 cdef	14.05 cd
5L2*(Y)*	13.00 cdefg	14.35 d
5L1*(Y)*	13.50 efg	14.50 d
10cL3*(Y)*	14.55 g	14.60 d
T(+)	14.10 fg	14.10 cd

(1) Fruits (n=10) were wounded, treated with 15μl of a suspension of antagonist (10^5 or 10^4 bacteria or yeast cells/ml) and inoculated with the pathogen (10^5 conidia/ml). *(B)*=bacteria, *(Y)*=yeast.
T(+)=control (fruits treated with water and inoculated with the pathogen.
(2) Means in a column followed by one same letter are not significantly different ($P_{0.05}$, tukey's multiple range test)

In vitro antagonism
In this test, 4 different interactions were observed (Table 2). Two did not express antagonism: the 2 colonies coexisted side by side without interacting

(type B) or the pathogen grew over the antagonist (type A). The two others expressed antagonism through a disturbance (type C) or an inhibition zone (type D) around the antagonist colony.
A mycelium-free inhibition zone between the antagonist and the pathogen was observed for 4 yeasts on PDA and malt agar and for 2 bacteria only on malt agar. The 4 other yeasts and 3 bacteria (the 2 previous and another one), disturbed the growth of the pathogen on malt agar and PDA but their activity depended on the medium.

Survival on leaf disks
All the strains except 2 yeasts survived until 21 days after inoculation on leaf disks (Table 3). The counts for yeasts were extremely homogeneous while those of bacteria were more variable.

DISCUSSION

Its seems strange that some antagonists (2 yeasts 5L1 and 5L2, and 1 bacteria 10B6) highly efficient in vivo (effective when inoculated at a concentration of 10^6 UFC/ml) were over-grown by the pathogen on PDA. But many authors have reported cases of great differences between in vitro and in vivo tests.
The variability in the counts of bacteria compared to those of yeasts suggests that yeasts maintain their population level more constant.

Table 2. IN VITRO ACTIVITY OF THE ANTAGONISTS, MEASURED AFTER 7 DAYS AT 19°C

Strains[1]	BEHAVIOUR OF THE PATHOGEN (2) (Types A, B, C, D) on	
	PDA	MALT AGAR
5B4*(B)*	C	D (2.0)
10B1'*(B)*	C	D (2.0)
5B4'*(B)*	C	B
10B6*(B)*	A	B
10cL4*(Y)*	D (2.7)	D (2.8)
5L3*(Y)*	D (3.5)	D (3.0)
10cL3'*(Y)*	C	C
10L2*(Y)*	D (3.5)	D (3.7)
5L2*(Y)*	A	C
5L1*(Y)*	A	C
10cL3*(Y)*	C	C
10L8*(Y)*	D (2.3)	D (2.3)

(1) *(B)*=bacteria, *(Y)*=yeast.
(2) The pathogen grew around and over the colony of the antagonist (Type A) ; it grew around the colony of the antagonist without interaction (Type B) ; the growth of the pathogen was inhibited around the antagonist with an inhibition zone <1mm or a mycelium less dense (Type C) ; an inhibition zone >1mm appeared (Type D).

Table 3. SURVIVAL OF THE ANTAGONIST ON DISKS OF STRAWBERRY LEAVES (cv. CAPITOLA AND FAVETTE)

Strains(1)	COUNTS OF MICROORGANISMS PER LEAF DISKS (2) (Log CFU)			
	Days after inoculation			
	3	7	14	21
5B4*(B)*	4.00+0.23	3.01+0.26	3.07+0.47	3.79+0.24
10B1'*(B)*	3.74+0.20	3.94+0.25	4.46+0.17	4.17+0.15
5B4'*(B)*	5.04+0.03	4.54+0.12	4.50+0.25	5.04+0.26
10B6*(B)*	4.39+0.14	2.50+0.19	4.21+0.49	2.74+0.40
10cL4*(Y)*	5.12+0.18	5.40+0.08	5.06+0.27	4.71+0.36
5L3*(Y)*	5.59+0.10	5.36+0.09	5.16+0.08	4.98+0.09
10cL3'*(Y)*	4.54+0.33	l.d.	l.d.	l.d
10L2*(Y)*	4.68+0.18	4.81+0.08	3.82+0.15	4.81+0.10
5L2*(Y)*	5.37+0.17	5.80+0.05	5.83+0.03	5.80+0.06
5L1*(Y)*	4.97+0.12	4.38+0.17	4.28+0.14	4.20+0.19
10cL3*(Y)*	4.48+0.26	l.d.	l.d.	l.d.
10L8(Y)	5.26+0.04	5.82+0.06	5.38+0.06	5.15+0.08

(1) *(B)*=bacteria, *(Y)*=yeast.

(2) Means are averaged values of 6 replicates + the standard error. Background microflora (non inoculated disks) were 4.5 - 5.5 bacteria and 4.5 - 5.0 fungi per disk.

l.d. limit of detection

CONCLUSION

We have isolated 4 yeasts (5L3, 10L8, 10L2 and 10cL4) and 2 bacteria (5L4 and 10B1') which have a good inhitory power in vivo and in vitro and survive on leaf disks for a long time. The 2 bateria were very efficient and one of them (5B4) is particulary promising.
The survival of the more efficient antagonists must be tested with high variations of relative humidity, or temperatures, more similar to the real conditions of the field.

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REDUCTION OF LOSSES DUE TO FUNGAL ROTTING DURING COLD STORAGE OF DUTCH WHITE CABBAGES BY POST-HARVEST TREATMENT WITH PSEUDOMONAS AND SERRATIA ANTAGONISTS

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Summary

Dutch white cabbages were dipped into bacterial suspensions of *Pseudomonas fluorescens* strain CL42, CL66, CL82, *Serratia plymuthica* strain CL43 or *Serratia liquefaciens* CL80 prior to cold storage in 3 individual trials.

Trial 1 and 2 were carried out in an experimental cold store at Manchester University with a temperature of between 4 and 6°C. Trial 3 was carried out in a commercial cold store at Geest Farms Ltd. (Spalding UK) with a temperature of between 1 and 3°C. In trial 1 cabbage heads of all treatments were sprayed with a suspension of *Botrytis cinerea* spores to provide extra fungal inoculum. No extra inoculum was applied in trials 2 and 3.

The amount of surface area covered by fungal growth was assessed at 6-weekly intervals during storage and the trimming losses were determined after 8-10 months. Only strains CL80 and CL82 were found to similar control to the fungicide treatment in all 3 trials. However, in the commercial cold store CL42 showed better results than any of the other bacterial strains. Except for trial 1, in which isolate CL82 gave significantly better control than the standard fungicide treatment, control was either similar to or lower than the fungicide treatment.

Introduction

Long term cold storage of Dutch white cabbages (*Brassica oleracea* L.) is common practice in many European countries to allow a constant supply of fresh vegetable to retailers and the processing industry. Cabbages are usually harvested before the first night frost in October/November and stored for up to 9 months. Storage temperatures in modern cold storage are at approximately 1°C and to avoid weight loss by dehydration the relative humidity in stores is usually high.

Under these storage conditions *Botrytis cinerea* and to a lesser extent *Alternaria* spp. are the major post-harvest diseases (Geeson, 1978; Brown *et al.*, 1975). Prior to the use of fungicides these organisms significantly reduced the time cabbages could be stored and could result in total loss of crops as early as 4 months after transfer into stores. The use of protective and systemic fungicides has greatly reduced losses (Brown *et al.*, 1975) and currently guarantees a constant supply of cabbages throughout the year. The use of fungicides, especially for postharvest disease control has, however, recently raised much consumer concern. This and the possibility of EEC legislation forbidding the post-harvest use of all fungicides has resulted in the search for alternative biological control methods.

We previously reported a survey on the microflora of Dutch White cabbage leaves and the isolation of antagonistic micro organisms from Dutch white cabbage surfaces using conventional plate assays on cabbage extract agar seeded with *Botrytis cinerea* spores (Leifert *et al.*, 1992a). This resulted in

the selection of approximately 100 antagonistic bacterial strains. We then developed a bioassay with heated leaf disks (Leifert et al., 1992b) and screened strains for their ability to suppress Botrytis cinerea and Alternaria brassicicola on leaf disks at low temperatures (Leifert et al., 1993). The 6 strains giving best control in leaf disk bioassays were then included in the storage trials described here.

Materials and Methods

Serratia plymuthica strain CL43, Serratia liquefaciens CL80 and Pseudomonas fluorescens strains CL42, CL66 and CL82 were used. Cabbage heads were dipped for into bacterial suspension containing 10^7–10^8 cfu/ml (see Leifert et al. 1993 for the methods used to prepare the bacterial inocula). In trial 1 cabbages were additionally sprayed with a conidial suspension of Botrytis cinerea containing 10^6 spores/ml. Cabbages were then transferred into plastic vegetable trays (12 cabbages/tray, 10 cabbages/tray for the fungicide treatment), sealed in a plastic bag to avoid cross-contamination between trays and weighed. Five trays (replicates) were used per treatment in each trial. Trays of trials 1 and 2 were incubated in an experimental cold store at Manchester University with a temperature of 4-6°C. Trays of trial 3 were incubated at a commercial cabbage cold store at Geest Ltd., Spalding, UK which had a temperature of 1-3°C. Individual cabbages were scored for the percentage surface area covered by fungal growth at 6 weekly intervals. When approximately 80% of the surface area of control plants were infected a destructive assessment was made. All infected leaves were removed and the weight loss was determined to determine the reduction in marketable value of the cabbages.

Results were analyzed by Analysis of Variance using the Epistat software.

Results and Discussion

Visible fungal infection started between 3 to 4 months after harvest and transfer of cabbage heads into storage. Spoilage of stored cabbages was more rapid in the two trials in Manchester which were stored at slightly higher temperatures then the trial carried out at Geest. The additional fungal inoculum applied in one of the Manchester trials also accelerated fungal spoilage.

There was, however, little variation in the effect most bacterial antagonists had in the 3 different trials. Of the 4 Pseudomonas and 2 Serratia strains tested only 2, P. fluorescens strain CL82 and S. liquefaciens CL80 gave significant control of fungal spoilage in all 3 storage trials (Table 1). Strain CL82 gave similar level of control to fungicide treatment in the 2 trials without extra fungal inoculum, and gave significantly better control than the fungicides in the trial with extra fungal inoculum. Strain CL80 also significantly reduced spoilage in all 3 trials, but was overall less effective than strain CL82. These two strains will therefore be studied further. Strain CL42 gave variable results. Protection was poor in the 2 trials at Manchester but very good under the cooler conditions in the commercial store at Geest Farms Ltd. (Table 1). This strain will therefore also be included in further studies. All other strains did not achieve satisfactory control and will not be tested further.

The results obtained in these storage trials did not correlate to the size of inhibition zones we observed on cabbage extract

Table 1. Percentage leaf area covered by fungal growth (SA) and percent weight loss (WL) of Dutch white cabbages which were treated with bacterial antagonists or the protective fungicide Rovral in 3 storage trials.

Treatment	Trial 1#		Trial2		Trial3	
	SA	WL	SA	WL	SA	WL*
untreated	82	34	82	32	65	26
Rovral	41	18b	18	13b	18	14b
Pseudomonas fluorescens						
CL42	52	25a	58	18b,c	23	15b
CL66	52	25a	ND	ND	57	23ns,c
CL82	13	13b,d	29	13b	25b	16b
Serratia plymuthica						
CL43	47	23b	63	27ns,c	58	23ns,c
Serratia liquefaciens						
CL80	25	18b	47b	17b	33	15b

extra fungal inoculum was applied
* estimated on the basis of the surface area results
ns not significantly different to untreated control
a significantly better protection than control (p=0.05)
b significantly better protection than control (p=0.001)
c significantly less protection than by fungicide (p=0.05)
d significantly better protection than by fungicide (0.05)
ND not determined

medium (Leifert et al. 1993). This is not surprising and has been reported frequently for various biocontrol agent (Wilson & Wisniewski 1989). More disappointing was, however, that the prevention of fungal spoilage in field trials also correlated poorly to the degree of protection measured in the bio-assays with heated leaf disks. It is likely that the heating treatment of cabbage disks which was required for rapid infection of the cabbage tissue by conidia of Botrytis cinerea has distorted the accuracy of the leaf disk assay, which we are therefore currently evaluating.

Acknowledgement

We would like to thank Geest Farms Ltd., Spalding, UK for their cooperation.

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POSTHARVEST BIOLOGICAL CONTROL OF *BOTRYTIS CINEREA* ON GERBERA

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Abstract
Preliminary experiments were performed to test the potential of different micro-organisms (*Bacillus brevis, Pseudomonas cepacia, P. aureofaciens, Cryptococcus laurentii, C. albidus and Trichoderma hamatum*) in controlling *Botrytis cinerea* on gerbera flowers during post-harvest. At a concentration of 10^7 cfu/ml and after a period of two to three days on the flower surface (relative humidity < 90%) some organisms can control *B. cinerea* as good as Rovral (iprodion). Residues on the flower surface or negative effects on the quality of the flowers have not been observed. Further research is needed to select the best antagonist, the optimal concentration and the best method for application and timing.

Introduction

Botrytis cinerea causes damage to cut flowers like gerbera, rose, chrysanthemum and potted plants such as Saintpaulia (De Jong, 1985). Necrotic lesions ('spotting') occur on flower buds and petals, and are caused by early infections. These symptoms are encouraged by a relative humidity above 93% (Salinas *et al.*, 1989), as when flowers are packed into boxes and rapid changes in temperature occur due to transfer from cold storage into trucks, and than back into store after harvest. The temperature during post-harvest fluctuates between 5 and 20°C. Between 18 to 25°C many lesions occur within 24 h of harvest (Salinas *et al.*, 1989). Control of *B. cinerea* on the flowers in the glasshouse is not possible, because in the glasshouse every day new flowers are developing and harvested. Usually *B. cinerea* is controlled during post-harvest, but this chemical control is not always effective. The aim of this study was to test the potential of *Bacillus brevis* (Edwards & Seddon, 1992), *Pseudomonas cepacia* (Janisiewicz & Roitman, 1988), *P. aureofaciens*, *Cryptococcus laurentii* (Roberts, 1990), *C. albidus* and *Trichoderma hamatum* (Nelson & Powelson, 1988) as a biological control agent (BCA) for *Botrytis cinerea* on gerbera flowers in a post-harvest treatment.

Materials and Methods

Gerbera flowers (cv. 'Terrafame') were grown on rockwool in an experimental glasshouse of 100 m^2. For all experiments isolate Bc-16 of *B. cinerea*, obtained from an infected gerbera flower was used. Flowers were inoculated with 1 ml suspension of *B. cinerea* ($1*10^4$ cfu/ml; approximately comparable to the highest infection pressure in the glasshouse) or with 1 ml suspension of a Biological Control Agent (BCA; $1*10^7$ or $1*10^8$ cfu/ml), in a Potter spray tower. With a Potter spray tower 10% of the suspension is actually sprayed on the flower surface (Potter, 1952). Per treatment 4

flowers were used and the experiments were repeated at least two times.
Flowers were inoculated with *B. cinerea* and dried for 10 minutes before placing them in a climate chamber at 75% relative humidity (RH) and 20° or 25°C. After 1 day the flowers were sprayed with one of the BCA's or Rovral (iprodion, 500 ppm). Only in the first experiment the BCA's were sprayed one day before *B. cinerea* was sprayed on the flowers.
After 0, 1, 2, 3 or 4 days at 75% RH the upper ten petals of each flower were placed on wet paper in a plastic box (>95% RH) and incubated at 20°C under fluorescent light. After one day at high RH, *B. cinerea* lesions on ten petals per flower were counted under a microscope. Five experiments were performed (Table 1).

Table 1. List of experiments.

Number Experiment	Conc. BCA (cfu/ml)	# Days BCA at 75% RH	Days at >95% RH	Temp. at 75% RH
1 First *B. cinerea*, then BCA	$1*10^6$	0	1	20°C
2 First BCA, then *B. cinerea*	$1*10^6$	1	1	20°C
3 First *B. cinerea*, then BCA	$1*10^6$	0 - 4	1	20°C
4 First *B. cinerea*, then BCA	$1*10^7$	0 - 3	1	20°C
5 First *B. cinerea*, then BCA	$1*10^6$	0 - 1	1	25°C

Results

If *B. cinerea* was sprayed on the flowers first, and the flowers were put in plastic boxes with a RH ≥ 95% immediately after spraying, then the BCA's did not have a good antagonistic effect on *B. cinerea* (Table 2). When a BCA was sprayed on gerbera flowers before *B. cinerea* the control of *B. cinerea* was significantly better (Table 2). However, in the field most of the spores are already on the flowers in the glasshouse before harvesting.
A few days of incubation (≥ 2 days) at 75% RH and a concentration of $1*10^7$ cfu/ml gave better results for all the BCA's tested. *B. brevis and P. aureofaciens* gave the same level of control as Rovral (Table 3, exp. 3; Table 4, exp. 4).
An incubation temperature of 25°C at 75% RH gave better results in controlling *B. cinerea* (Table 5). But this higher temperature is rare during postharvest. This result shows that the temperature can have significant effects on the antagonistic abilities of the BCA's.

Table 2. Effect of biological control agents on mean numbers of lesions per ten petals caused by *Botrytis cinerea*. The treatment *B. cinerea* is set at 100%. Means in each collumn followed by a common letter are not significantly different (P≤0.05).

Treatment	Number of lesions First Bc, then BCA Experiment 1	Number of lesions First BCA, then Bc Experiment 2
Botrytis cinerea	100 de	100 c
Bacillus brevis	104 e	92 c
Cryptococcus albidus	136 f	62 c
F5 (yeast)	91 bc	52 b
Pseudomonas aureofaciens	84 b	37 a
Pseudomonas cepacia	96 cd	54 b
Trichoderma hamatum	94 cd	119 d
Rovral (500 ppm)	55 a	34 a

Table 3. Effect of biological control agents ($1*10^6$ cfu/ml) on mean numbers of lesions per ten petals caused by *B. cinerea*, after different days of incubation at 75% RH and 20°C. The treatment *B. cinerea* is set at 100%. Means in each collumn followed by a common letter are not significantly different (P≤0.05).

Treatment	Numbers of lesions (experiment 3)				
	Day 0	Day 1	Day 2	Day 3	Day 4
Botrytis cinerea	100 b	100 c	100 b	100 b	100 b
Bacillus brevis	126 b	105 c	87 ab	79 ab	93 b
Pseudomonas aureofaciens	117 b	81 bc	75 ab	66 ab	49 a
Pseudomonas cepacia	102 b	79 bc	83 ab	71 ab	62 a
Trichoderma hamatum	105 b	71 ab	65 a	94 b	56 a
Rovral (500 ppm)	68 a	52 a	57 a	63 a	35 a

Table 4. Effect of biological control agents ($1*10^7$ cfu/ml) on mean numbers of lesions per ten petals caused by *B. cinerea*, after different days of incubation at 75% RH and 20°C. The treatment *B. cinerea* is set at 100%. Means in each collumn followed by a common letter are not significantly different ($P \leq 0.05$).

Treatment	Numbers of lesions (experiment 4)			
	Day 0	Day 1	Day 2	Day 3
Botrytis cinerea	100 b	100 d	100 d	100 d
Bacillus brevis	65 a	37 a	28 a	24 a
F5 (yeast)	107 bc	109 d	62 c	65 c
Pseudomonas aureofaciens	48 a	39 a	49 b	57 c
Pseudomonas cepacia	54 a	45 ab	27 a	28 a
Trichoderma hamatum	100 b	58 c	45 b	91 d
Rovral (500 ppm)	52 a	51 bc	33 a	40 b

Table 5. Effect of biological control agents at 25°C on mean numbers of lesions per ten petals per flower caused by *B. cinerea*. The treatment *B. cinerea* is set at 100%. Means in each collumn followed by a common letter are not significantly different (experiment 5; $P \leq 0.05$).

Treatment	Number of lesions Day 0	Number of lesions Day 1
Botrytis cinerea	100 d	100 d
Bacillus brevis	33 b	25 b
Cryptococcus laurentii	32 b	17 ab
Pseudomonas aureofaciens	73 c	26 b
Pseudomonas cepacia	58 c	24 b
Trichoderma hamatum	33 b	43 bc
Rovral (500 ppm)	14 a	8 a

Discussion

There are possibilities for BCA's in postharvest biological control of *Botrytis cinerea* on gerbera, especially for *B. brevis and P. aureofaciens*. A concentration of $1*10^7$ cfu/ml and 2-3 days in a climate chamber (RH≤90%) is necessary for a good control of *B. cinerea*. Residues on the flowers or negative effects on the quality of the flowers have not been observed.
More research is needed on the effect of lower temperatures (5-15°C, postharvest conditions) and lower and higher relative humidities (between 50 and 90%) on the effectiveness of BCA's. Also the effect of growing media on the antagonistic possibilities of the BCA's needs more research.

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POTENTIAL FOR POST-HARVEST BIOLOGICAL CONTROL OF ANTHRACNOSE OF BANANAS

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Summary

The yeasts, *Hyphopichia burtonii* and *Candida guilliermondii*, and the filamentous fungus, *Talaromyces flavus*, were screened for their ability to antagonise, or inhibit growth of the anthracnose pathogen, *Colletotrichum musae*. Over a range of water availabilities and temperatures the yeasts were ineffective at antagonising or inhibiting germination or growth of *C.musae* on low and high nutrient status media. *T.flavus* inhibited growth of *C.musae* at a distance, and continued to grow over and colonise *C.musae* mycelium over a range of environmental conditions. *T.flavus* produced extracellular metabolites when grown on cellophane discs which significantly inhibited spore germination and growth of *C.musae* over a range of conditions on both low and high nutrient status media. Tests are now in progress with cultures and culture filtrates of *T.flavus* to determine the ability of the biocontrol agent to inhibit or delay lesion formation on ripening banana fruit surfaces.

Introduction

Anthracnose and crown rot of bananas caused by *Colletotrichum musae* severely affects fruit quality and results in significant economic losses in many parts of the tropics. The spores of the pathogen infect green fruit forming a latent (quiescent) infection. After harvesting and transport to the markets, characteristic lesions occur on the fruit surface as the bananas ripen (Jefferies et al., 1990). Because of resistance to some commonly used fungicides used as post-harvest dips to control the disease, alternative methods for control including non-toxic coating materials and biological control agents are being sought. Any treatment which could delay the lesion expression or the rate of symptom development could be potentially useful for increasing shelf life. The objectives of this project were to screen a filamentous fungus *Talaromyces flavus*, and two yeasts, for their ability to antagonise and inhibit this pathogen in vitro over a range of environmental conditions and to determine their potential for controlling or delaying lesion expression on bananas.

Materials and Methods

Media

Potato dextrose agar (PDA), 1% malt extract agar (MEA) and 2% water agar + 0.5% glucose (WA) were used in this study. The water activity (a_w) of these media was modified to 0.99-0.93 by the addition of NaCl (Lang, 1967) and as detailed by Whipps and Magan (1987).

Growth and maintenance of fungi

Talaromyces flavus (*Penicillium vermiculatum*), and the yeasts *Hyphopichia burtonii* and *Candida guilliemondii* var *guilliermondii* were all isolated from mouldy agricultural substrates and maintained on PDA and MEA, respectively. The pathogen *Colletotrichum musae* was isolated from bananas in the West Indies (IMI 172697) and obtained from the International Mycological Institute.

Competition

Colony competition and interaction were studied using the method described by Magan and Lacey (1984). A numerical score was given to fungi based on whether they intermingled freely (Reaction A; 1); inhibited each other on contact or at a distance (Reaction B, C; 2, 3); one fungus inhibits the other on contact or at a distance and continues to grow through the inhibited colony (Reaction D, E; 4, 5). Generally, yeasts were streak inoculated 5 mm from the edge of one side of five replicate Petri plates or inoculated with a 4mm agar disc from the growing margin of a *T.flavus* colony. One day later, an agar disc of *C.musae* was placed diametrically opposite, 5 cm from the antagonist inoculation point. The growth of *T.flavus* and *C.musae* was measured at regular intervals for up to 30 days. Two measurements were made: R1, the furthest diametric distance grown by the pathogen (a control value) and R2 the distance grown on a line between the pathogen and antagonist inoculation positions (an inhibition value). Competition was also examined visually, to give a numerical value to each individual interacting fungus. The interacting mycelium after contact between *T.flavus* and *C.musae* was examined by removal of rectangular sections, mounting on a slide and staining as detailed previously (Magan and Lacey, 1984).

Effect of *T.flavus* and yeast metabolites on growth of *C.musae*

The method used was based on that of Gibbs (1967). The PDA or WA media modified to different a_w levels in Petri dishes were covered with cellophane (British Cellophane Co., PT600) and centrally inoculated with 4mm agar discs of *T.flavus* from the growing margin of PDA plates or streak inoculated with a loop of yeast suspension. The experiment was carried out with three replicates per a_w treatments and at 25 and 30°C. The plates were incubated for 12 days before the cellophane and the *T.flavus* colony were carefully removed. The agar media were then centrally inoculated with 4mm discs of the pathogen, *C.musae*. They were then incubated for up to 12 days. The diameter of the colonies were then compared with that of control plates.

Similarly, after removal of the cellophane and *T.flavus* or yeast colonies, 0.1ml of a 10^5 spores ml suspension was spread on the surface of three replicate plates per a_w and temperature treatment. Three 18mm discs were removed at random after 24 and 48hrs placed on slides, stained with lactophenol/cotton blue covered with a cover slip. Two groups of 50 spores per disc were examined for germination and compared with that occurring on control plates. Spores were considered to have germinated when the germ tube length was equal to or greater than the spore diameter.

Results

Table 1 shows that regardless of temperature or a_w, *T.flavus* inhibited *C.musae* at a distance and continued to slowly grow through and colonise the pathogen colony on both PDA and WA. By contrast the yeasts had no effect on growth of C.musae. The pathogen grew through the yeast colonies, sometimes forming characteristic pink conidial pustules in the yeast colony.

Table 1. Effect of a_w on numerical index of interacting fungi on both PDA and WA.

		Numerical interaction score			
Water activity		0.995	0.99	0.98	0.96
Temp.	Species				
15°C	*T.flavus - C.musae*	5 - 0	5 - 0	5 - 0	5 - 0
25°C	*H.burtonii - C.musae*	0 - 4	0 - 4	0 - 4	0 - 4
30°C	*C.guilliermondii - C.musae*	0 - 4	0 - 4	0 - 4	0 - 4

Table 2 shows the mean diametric growth (mm) of *C.musae* and *T.flavus* in relation to *T.flavus*: R1 - greatest radial growth (control value); R2 - diametric growth between inoculum positions, with in parentheses, % inhibition. Measurements after 15 days at 25 and 30°C are given.

Table 2. Comparison of diametric growth of interacting fungi at different water activities on two media with, in parantheses, percentage inhibition.

	25°C							
Water activity	0.995				0.98			
Medium	PDA		WA		PDA		WA	
	R1	R2	R1	R2	R1	R2	R1	R2
T.flavus	25	25	13	15	30	33	26	28
C.musae	85	45(47)	85	56(34)	85	46(46)	80	46(42)
	30°C							
T.flavus	30	30	20	22	37	32	31	32
C.musae	76	42(45)	65	57(13)	73	30(41)	65	41(37)

The two yeasts had no effect on spore germination or growth of *C.musae*. However, metabolites produced by *T.flavus* significantly affected both germination of conidia and mycelial growth of *C.musae*. At both 25 and 30°C and 0.995,0.98 and 0.96 a_w germination of practically all conidia was inhibited for 24hr. However, after 48hr, almost all conidia germinated at 25°C and all a_w treatments, but germ tubes were short and conidia swollen in appearance. Control spores had germinated and produced microcolonies by this stage. The effect of metabolites in the media on mycelial growth is

shown in Table 3. This shows that for 12 days growth of *C.musae* was significantly inhibited on both PDA and WA under all the test conditions. However, effectiveness was less marked on WA than PDA.

Table 3. Effect of metabolites of *T.flavus* on growth (mm,colony diameter) of *C.musae* at 25 and 30°C on PDA and WA after 12 days.

Water activity			0.995		0.99		0.98	
Medium	PDA*	WA*	PDA	WA	PDA	WA	PDA	WA
25°C	85	85	11	70	3	30	N.G.	35
30°C	85	85	10	36	N.G.	30	N.G.	34

* control colony diameter after 5 days.
N.G., no growth.

Infection of bananas with *C.musae* for 24-48hr and subsequent spraying with culture filtrates of *T.flavus* resulted in approximately 45-50% control of lesion expansion after 10 days incubation at 25°C.

Discussion

This study has shown that potential exists for using *T.flavus* for the biological control of the anthracnose pathogen, *C.musae*. Over a range of temperatures and a_w levels *T.flavus* was able to both antagonise and colonise hyphae of *C.musae* and inhibit growth at a distance. The xerotolerant yeasts (Magan and Lacey, 1986) screened in this study were ineffective, although other such yeasts, e.g. *Debaryomyces hansenii*, have been found to effectively control *Penicillium digitatum* on grapefruit (Droby et al., 1989). The ineffectiveness of the yeasts may partially be due to their inability to produce metabolites which could inhibit germination and growth of *C.musae*. Previously, yeasts such as *D.hansenii* have predominantly been found to act by effective competition for nutrients.

T.flavus has previously been found to suppress *Verticillium* wilt of eggplant (Marois et al., 1982) and to parasitise sclerotia of *Rhizoctonia solani* and sclerotia and hyphae of *Sclerotinia sclerotiorum* (McLaren et al., 1982). Marois et al. (1986) also found that *T.flavus* could effectively occupy the rhizosphere of a range of plants and reduce germinability of *Verticillium dahliae* microsclerotia when applied as a ascospore drench.

Further work is now needed to determine the concentrations of *T.flavus* conidia and ascospores to control lesion expansion of anthracnose on bananas and to characterise the metabolites with efficacy against this economically important tropical pathogen.

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BIOLOGICAL CONTROL OF POST-HARVEST DISEASES. GENERAL DISCUSSION.

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The session on biological control of post-harvest diseases showed that this rather new subject of biological control has expanded the last few years. The reports covered work on fruits, vegetables and flowers, and the storage experiments were performed under both laboratory and commercial storage conditions. Application of antagonist were done both into wounds under laboratory conditions and by application of the antagonist to a commercial pacing house line.

For fruits and vegetables there are some concerns on the use of antagonists directly on the edible produce. This has to be taken into consideration when selecting antagonists for post harvest treatments. What kind of antagonist should be used? Antagonists that produces antibiotics were argued against for several reasons. Due to the long tradition in using yeasts in the food industry, there were strong arguments on the advantage of using yeasts that were not able to grow at 37°C as biocontrol agents. But bacteria and filamentous fungi may also have advantages and should not be excluded in the development of post harvest biocontrol.

Many of the reports showed a clear correlation between the level of biocontrol agents (BCA) applied and the achievements. Unfortunately to obtain good effect the amount of BCA needed in many trials is to high to be used commercially.

Due to legislation in some countries, application of agrochemicals or BCA post harvest is not permitted. In such cases, application before harvest is a possible solution, but the advantages of a targeted application on the harvested product are then lost.
Mechanism of action of the BCA in post harvest biocontrol, is in most cases not proven, but competition for nutrients on wounded surfaces certainly play an important role in many cases. Janisewich (this issue) has clearly demonstrated the marked effect of different nutrients added with the biocontrol agent. Optimal use of nutrient

added together with the antagonist is therefore an important research field.

In long time post harvest storage, there is an increasing use of wax treatment of the products before storage. A combination of treatment with biocontrol agents and then wax has the possibility to create selective advantageous conditions for the biocontrol agent and should therefore be further investigated.

Should the BCA´s be applied as pure cultures or as mixtures of different antagonists? There are different experiences on this subject, some have obtained better control with mixtures of different organisms than each of them singly, but others have not seen any positive effect of mixtures. In post harvest biocontrol, when the environmental conditions can be controlled, the advantage of a mixture should be less than in other situations. There were also other arguments against mixtures from the commercial side, because the cost of commercialization of a mixture will be much higher than a pure culture BCA.

Formulation of BCA for post harvest treatment has been little investigated, but there was a general agreement that formulation also is of great importance in the biocontrol of post harvest diseases.

One of the worst ecological bottlenecks in biocontrol of cold stored products are the difficulties in finding antagonists that can be sufficiently active at cold temperatures. Trials have showed that it is possible to select effective cold tolerant antagonists, and there will be much to gain by putting more effort into selection of antagonists especially selected for biocontrol activity on the product under commercial storage condition.

Biological control of post harvest diseases has several advantages over biological control in the field situation. The presentation and discussions in this workshop showed that the progress in this field is impressing, and post harvest biological control will probably be one of the fields with the greatest commercial interest in the future.

Ecology of biocontrol agents

INTRODUCTION: ECOLOGY

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In 1957, Newhook published a paper on "the relationship of saprophytic antagonism to control of *Botrytis cinerea* Pers. on tomatoes". This paper demonstrated that inoculation of dead petals with *Cladosporium herbarum* and *Penicillium* sp. reduced fruit rot almost completely. If presented at this workshop, this work would have been one of the major contributions. The limited progress in our field of research in the following decades might be caused by the lack of economic pressure because of the easy and effective control by fungicides but also by the absence of fundamental knowledge on the microbial ecology of the phyllosphere in its broadest sense. Such scientific knowledge is a prerequisite for successful exploitation of naturally occurring or introduced antagonists. Scattered information on this subject has been exchanged regularly since 1970 at international phyllosphere symposia. The proceedings of these symposia comprise our basic texts.

The effect of biotic and abiotic factors on the phyllosphere microflora should be known in order to estimate whether adequate antagonistic densities of the microorganisms can be reached under practical conditions. Methods for quantitative monitoring are still largely based on tedious plating techniques with generally little qualitative distinction. Nevertheless, after introduction of the antagonist, its population density should be monitored because this is the only way to discover whether a possible failure of control is caused by inadequate population densities or lack of expression of antagonism. We should also be aware that control may not be recognized because of the development of naturally occurring antagonists in the control treatment.

In this chapter first the role of naturally occurring antagonists will be discussed. Their importance in disease control is difficult to demonstrate under field conditions, because agrochemicals being the main tools to create prolonged differences in population densities have direct effects on pathogen populations. Indirectly, however, it could be demonstrated that elimination of yeasts by fungicides resulted in a accumulation of infection-stimulating nutrients, such as aphid honeydew. Knowing that phyllosphere yeasts and other saprophytes have the potential of moderating infection by necrotrophs, it is important to know to what extent different agrochemicals affect their population densities.
Secondly, attention will be paid to ecological criteria for successful colonization of the substrate after introduction of the antagonists and in competition with the native microflora. A molecular method will be described to identify antagonistic Trichoderma spp. after release.

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MICROBIAL COLONIZATION: FROM SAMPLING TO SIMULATION.

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Introduction

In research on biological control, microbial colonization is an important issue. In this paper we discuss two different quantitative aspects of microbial colonization that deserve attention: i.e. sampling and simulation. Sampling is usually done with one or more of the following goals:
- to assess whether the biological control agent (BCA) can survive and establish itself on inoculated plant parts,
- to assess whether the BCA spreads to non-inoculated plants or plant parts,
- to assess disease severity or incidence, with the aim of establishing the effect of the BCA.

For a good evaluation of the prospects of a given BCA, it is important to know its population dynamics and its effect on disease as accurately as possible. The accuracy of the mean values that are determined through sampling can be strongly influenced by the sampling plan that is being used. The way an experiment is sampled, however, is often governed at least partly by constraints in time and labour. In the first part of this paper we discuss several aspects of sampling, including sampling in the presence of spatial patterns.

Once reliable quantitative data on microbial colonization are available, these data can be used in the development and validation of a simulation model. In the second part of this paper, we describe a simulation model that was developed for the population dynamics of yeasts on wheat leaves and discuss how simulation models can be used in biocontrol research.

Sampling

Sampling plays a number of roles in a study of a BCA. It may be used to simply estimate a mean population size, to compare two or more treatments, or to investigate the possible presence of a spatial pattern. In planning a sample, the first two goals require an estimate of the variance of observations. Previously obtained data, or a small preliminary experiment, can help determine this. However, we caution against the use of bulk samples, wherein data from several plots are combined before analysis, since this can result in a misleading estimate of variability (Kinkel, 1992).

If the goal of a sample is to estimate the mean population size, say, of a BCA, then it may suffice to calculate a confidence interval for the mean. The width of the confidence interval depends on the variability of observations and on the sample size (Cochran, 1977). By using a preliminary estimate of variance, the size of the planned sample can be determined such that the resulting confidence interval will have (at least approximately) the desired width.

In most experiments sampling is conducted to compare two or more treatments, and in that setting a power calculation is of value. We must recognize that in a small experiment with large variability, it might happen that real differences between treatments would go undetected. Such experiments are said to have low power, where, roughly speaking, power is the probability of detecting a significant difference between two treatments. A power calculation is thus done to try to determine the appropriate number of replicates to provide a good chance of detecting significant differences. Using formula (6.14.4) provided in Snedecor and Cochran (1989), one starts with a desired power (i.e. probability), the estimated variance between plots, and expected difference between treatments. The power calculation then results in the number of replicates that will provide the desired power. Alternatively, a power calculation can be used to assess the sensitivity of an experiment: given the maximum number of replicates that fits into a field or greenhouse

experiment (or budget), we can calculate the size of difference between treatments we can expect a reasonably high chance of detecting. In the extreme, this can be used to determine whether a particular experiment is worth conducting.

The number of time points at which to sample is strongly dependent on the purpose of the experiment and on the disease and BCA that is being sampled. Some diseases are most destructive on young plants, whereas for other diseases we are only interested in their effect on the harvested product. Therefore, no general remarks can be made about the number of time points, except that obviously the experiment must be designed so that the time points cover the biological stages of interest. We would like to add however, that in case of sampling microbial populations the time of day is important, because populations can change during the course of a day (Hirano & Upper, 1986).

Once the sample size, number of replicates and number of time points have been decided, the next choice is where and how to sample. Most often, a simple random sampling plan will be used, without taking into account a possible non-random spatial distribution. However, the existence of a spatial pattern in a population being sampled can influence the efficiency of a sampling strategy: estimates of the mean may be excessively variable if an inappropriate sampling plan is used. Consequently, it will be desirable to develop sampling strategies that take the spatial patterns into account. Knowledge of the spatial distribution of the population or disease to be sampled is therefore indispensable.

Spatial patterns can occur on several scales, and across dimensions. For example, there may be patterns of BCA populations within a given leaf. It is equally likely that there are spatial patterns vertically within a given plant. This might occur particularly on plants with distinct leaf layers. In disease ratings and population studies, this vertical spatial pattern is usually taken into account by either sampling one particular leaf layer or by keeping the results for different leaf layers separate. Furthermore, there may be horizontal spatial patterns between plants. This kind of pattern has only recently been recognized as an important factor in plant pathological research. So far, most studies on spatial patterns have focussed on disease. Several methods to determine spatial patterns exist, such as quadrat methods, distance methods and autocorrelation approaches. The merits and disadvantages of quadrat and distance methods have been discussed previously (Clayton & Hudelson, 1991), and for the rest of this discussion we want to focus on autocorrelation approaches. Autocorrelation can be applied to plants, as in the example we will discuss, but also to quadrats. To apply autocorrelation methods on the plant level, we have to be able to quantify disease or populations on every plant in, for example, a row segment. Autocorrelation analysis is based on the premise that disease or populations on a given plant may be influenced by disease or populations on neighbouring plants. The value of the autocorrelation function at lag *s* is the correlation of disease values or population densities for all pairs of plants, *s* plants apart. Autocorrelation functions are used in autoregressive moving average (ARMA) models, which in some sense summarize the autocorrelation function and provide a description of (but probably not the mechanism for) the relationship between disease or populations on adjacent plants. An ARMA (p,q) has the form:

$$Y_t = \phi_1 Y_{t-1} + \ldots + \phi_p Y_{t-p} + \epsilon_t - \theta_1 \epsilon_{t-1} - \ldots - \theta_q \epsilon_{t-q} + \delta$$

in which Y_t corresponds to the amount of disease or the population density for the plant at position t in the row and the ϵ's represent random noise components associated with each plant.

Our recent work has examined the use of ARMA models with the system bacterial brown spot on snap bean plants. Work by Hudelson et al. (1989) showed that disease incidence consistently shows non-random patterns which can adequately be described by ARMA models. This and other examples (see Clayton & Hudelson (1991) for references) show that disease often follows a non-random distribution. However, not only disease, but also microbial populations can show spatial patterns. In our research on the causes of the spatial patterns in brown spot incidence in snap beans, we studied populations of the causal agent *Pseudomonas syringae* pv. *syringae*. In field experiments in 1991 and 1992, we estimated *Pseudomonas syringae* populations with the ice-nucleation test (Hirano et al., 1987). In 1991, we found non-random patterns in naturally occurring populations on snap bean leaves. The patterns were similar to the

ones that were found for disease incidence. In both years, experiments were performed in which the bacteria were put on bean seeds or sprayed on the leaves three weeks after planting. Disease incidence showed non-random patterns in all treatments, which suggest non-randomness in inoculum (Rouse et al.,1985). In three experiments, all bean seeds in 5 m row segments were dug up, both from a control treatment and from the seed-inoculation treatment and populations were estimated either by plating or with the ice-nucleation test. In the seed-inoculation treatment, non-random patterns were found starting 24 h after planting, whereas in the control treatment these patterns started to appear several days after planting, but before emergence. The patterns could be described by similar ARMA models as were found before for disease incidence. Data from day 0 suggest that the inoculum was put on the seeds randomly, so the patterns occurred within the first few days, either by non-random growth or survival of the bacteria. Since the patterns appear in the control when the populations are increasing, non-random growth seems the most likely explanation. Populations were also estimated on all leaflets of every plant in 5 m row segments. The median freezing temperature per plant showed non-random patterns in both the inoculated and control treatment. An example is shown in Fig.1.

In the presence of such spatial patterns, it can be demonstrated that a random sampling plan could give a larger variance of the estimated mean than a random start systematic sampling plan (Cochran, 1977). In a random start systematic plan, a random starting point is chosen, and then every *k*th plant is sampled thereafter, where *k* is a value to be determined. Clayton & Hudelson (1991) give an example where the standard error of the sample mean would be twice as high in random sampling as in a systematic sample. In addition to their added precision, systematic sampling plans are easier to implement, and thus can provide a benefit in terms of time and labour constraints. (Research on aspects of systematic sampling plan implementation is underway).

Systematic sampling plans can also be used to detect spatial patterns. Several modifications of systematic sampling for this purpose were developed by Clinger and Van Ness (1976) and by Hudelson (1990). These sampling plans are particularly useful for detecting spatial patterns on a larger scale. These plans allow the calculation of an autocorrelation function even though only a fraction of the plants are sampled. Moreover, they permit sampling up to 100 m row segments in a single day. Therefore, as with the use of systematic sampling to estimate means, time and labour costs are not necessarily higher for the use of systematic sampling to detect and quantify spatial patterns.

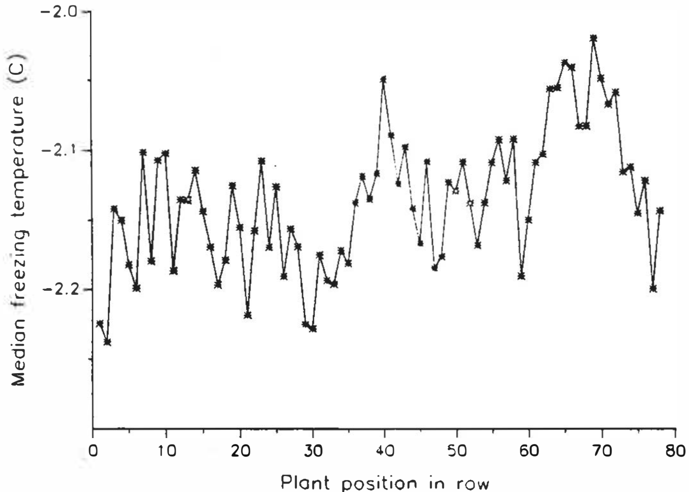

Fig.1. Median freezing temperatures of all leaflets per plant as an estimate for *P. syringae* population densities (Hirano et al., 1987) for individual snap bean plants within a 5 m row segment. The data were collected in an untreated control plot in a snap bean field in Wisconsin, 30 days after planting in 1992.

Simulation

The second quantitative aspect of microbial colonization that we want to discuss is simulation. Basically, simulation models comprise three kinds of variables, i.e. state variables, rate variables and independent variables. We will illustrate the use of simulation models in biocontrol research with a model which simulates the population development of naturally occurring yeasts on wheat leaves and the amount of aphid honeydew consumed by the yeasts (Dik, 1991). In this model, the population density of the yeasts and the aphids and the amount of honeydew are the state variables. The rate variables are the rates with which these state variables change, and the independent variables are the factors that influence the rate variables, for example temperature, vapour pressure deficit, rain and developmental stage of the crop. Every timestep (1 h) the state variables are adjusted and new rates are calculated. For estimation of parameter values, data from the literature and from growth chamber experiments, specifically designed for this purpose, were used. The model was validated with field experiments on two locations in two years (Dik, 1991).

Simulation models for biocontrol agents can be useful tools. The model for the yeasts, for example, can be used to evaluate the effect of the yeasts in different situations, such as different growth rates of the aphid populations. So far, this is mainly done to verify possible explanations for observations in the field. For example, in one field experiment, honeydew consumption by the yeasts could not keep up with the honeydew production, so honeydew accumulated, whereas in a simultaneous experiment with a different aphid population development this accumulation did not occur. With the model, the assumption that the difference between the two experiments was caused by the different aphid populations can be verified. Apart from evaluating the role of naturally occurring BCA's, the model might also be used to evaluate the effect of applying a BCA to the plants. In the above mentioned situation, the model indicates that applying yeasts would have prevented the accumulation of honeydew. Also, the effect of applying a fungicide that decreases the yeast population could be simulated. In the future, a model like this may be used in combination with a model that simulates aphid population development and yield loss by honeydew deposition and suction damage. The role of the yeasts in reducing honeydew damage can be estimated by the model, and could help to improve calculation of the damage threshold.

To build a simulation model, reliable quantitative data are needed. Most of the experiments described in the literature have been done with a different goal, and are therefore often not sufficient to build a model. Thus, additional data have to be collected. The data that are used for the validation of the model have to come from separate experiments, preferably large scale experiments or observations in natural systems. Although building a simulation model requires some extra work, we think such a model can be very useful in research on biological control in several ways. It offers a good method of evaluation of the effect of naturally occurring antagonists. It also offers the possibility of using results from laboratory experiments to predict the survival and effect of applied antagonists (Knudsen & Hudler, 1987). This means that field experiments can be limited to antagonists that are likely to be able to survive and be effective in the field. Another advantage of the use of simulation models is that they show which parts of the system are sensitive to relatively small changes in the inputs and assumptions. This can lead to additional research directed to improve the knowledge of these parts of the system. A model for a BCA that is applied to the plants can help to improve the timing and rate of application. Especially for timing of the application, simulation models for the disease against which the BCA is directed, can be helpful.

Models are usually influenced by variables like temperature and vapour pressure deficit. In the field, these are hard to predict. This seems to be one of the problems in using simulation models to predict disease. However, in the case of microbial populations, models can be used to calculate population densities up to the present day, given the weather up to the present day. This means a reduction in sampling, and the possibility of a more instantaneous reaction.

So far, simulation models rarely have a spatial component. We want to point out however, that if known factors that cause non-random population growth are included, or if the mechanism of dispersal of the microorganism that

is simulated is known, building it into a model would help predict spatial distribution. This in its turn will give us an indication on how to develop a sampling plan.

Conclusions

Sampling and simulation are related to each other in research on microbial colonization: the detection of spatial patterns by sampling may lead to insights in the ecology of the microorganism of interest, which can in turn be used in a simulation model. On the other hand, a simulation model may give an indication of the spatial distribution that can be expected and can help in the development of a sampling plan. Separately, both a well-adjusted sampling plan and the use of simulation models may improve accuracy and efficiency of biocontrol research.

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DYNAMICS OF MICROMYCETA POPULATIONS IN THE APRICOT-TREE PHYLLOPLANE AS RELATED TO THE COMPLEX OF ABIOTIC AND BIOTIC FACTORS

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Summary

The apricot-tree phylloplane is populated by a parasitic and saprophytic microflora from bud swelling up to leaf fall, represented by bacteria, actinomyceta and micromyceta. The pathogens of the phylloplane have their own microparasites, saprophytic fungi playing an antagonistic role, while some hyperparasites exert a potential control of pathogens.

The micromyceta species with antagonistic role identified by us in the apricot-tree phylloplane were: *Fusarium lateritium*, *Chaetomium globosum*, *Trichothecium roseum*, *Trichoderma viride* and *Gliocladium roseum*, as well as some hyperparasitic fungi: *Gonatbotrys simplex*, *Cladosporium uredinicola*, C. *sphaerospermum*, *C. herbarum*, *Acremonium alternatum* and *Aphanocladium album*.

In various apricot-tree phenophases the colonies of these fungi vary quantitatively and qualitatively in the phylloplane, as depending on climate conditions and treatments applied to control the specific pathogens.

Introduction

On the surface of healthy leaves of various plants there is a rich and varied microflora, represented by bacteria, actinomyceta and micromyceta. This microflora plays an important role in the evolution of processes of contamination and infection with pathogens, as well as on some physiological processes of plants (Andrews and Kinkel, 1986; Sundheim, 1986; Fokkema, 1992). In the phylloplane saprophytic fungi develop, having a competitive action with pathogenic fungi, hampering their activity, or on some other saprophytic species provided with high and rapid propagation ability. Populations in the phylloplane suffer quantitative and qualitative changes during the plant vegetation period, these being caused by climate factors, plant development phases and pesticides applications (Skajennikoff and Rapilly, 1981; Drăgoescu *et al.*, 1985; Baicu and Oprea, 1990; Fokkema, 1981, 1983; Pennycoock and Neewhock, 1981). Nevertheless, it was noted that phylloplane alternations due to sprays designed to control parasites have a limited duration, the populations rapidly recovering. Influences can have longer duration only when applications succeed at short intervals (Oprea and Baicu, 1988).

Our investigations aimed at establishing the influence of abiotic and biotic factors on microflora structure in the apricot-tree phylloplane during a whole vegetation season (Baicu and Oprea, 1991). This concern was focused on influence of fungicides used to control the foliar pathogens of the apricot-tree.

Research will continue and will be amplified in relation with intensifying the integrated control in orchards.

Material and method

Trials have been effected under laboratory conditions and in the R.I.P.P. orchard in Bucharest where treatments were applied onto cv. Meilleur d'Hongarie.

Micromyceta evolution was pursued from budding up to complete leaf fall (on buds, floral components, young and senescent leaves, tissue fragments collected from the foliar scar zone). For identification of micromyceta in the phylloplane 7 mm diameter disks were detached from various leaf zones, these being then placed in Petri dishes on a water-agar medium. Fourteen days later 100 disks of each variant were examined and colonies were counted for each species. Similar procedures were also used for the study of anthoplane or tissue fragments extracted from their foliar scar.

The same methods have been applied to assess the phylloplane changes resulted from season applications to control some apricot-tree pathogens (*Monilia laxa* and *Stigmina carpophila*).

Leaves have been collected after 2 hrs. 2, 4 and 8 days from the treatment applied at fruit formation. The fungicides used in all 3 variants are exposed in table 1.

Table 1 Fungicides used for controlling the main diseases of apricot-trees

Compound	Formulation	Active ingredient	Conc. (%)	Producer
Topsin M 70	WP	Methyl thiophanate	0.1	Nippon Soda Japan
Captadin	WP	Captan	0.2	Borzešti Romania
Bayleton 25	WP	Triadimephon	0.2	Bayer Germany

Results

The following saprophytic species were present in the phyllosphere in various phenophases, from bud swelling up to the end of leaf fall (from February through November): *Cladosporium herbarum*, *C. sphaerospermum*, *C. uredinicola*, *Aureobasidium pullulans*, *Trichoderma viride*, *Gonatobotrys simplex*, *Chaetomium globosum*, *Aphanocladium album*, *Oospora* spp., *Gliocladium aureum*, *Acremonium alternatum*, *Trichothecium roseum* and *Fusarium lateritium*.

Occurrence of these species varied with the apricot-tree phenophase (table 2). On winter bud scales species from the genera *Cladosporium* (*Cladosporium herbarum* and *C. sphaerospermum*), *Acremonium alternatum*, *Oospora* spp. and *Aureobasidium pullulans* prevailed. At bud opening, besides these species, *Trichothecium roseum* and *Fusarium lateritium* also developed. During flowering the number of fungus species increased in the anthoplane, new species being established: *Gonatobotrys simplex* and *Trichoderma viride*. This composition maintained throughout the season till autumn, when leaves entered a new natural phase of senescence. The number of species decreased, and also their frequency, depending on the pathogens established in that period in the phylloplane; during a heavy attack by *Tranzschelia pruni-spinosi* in September 1988, *Cladosporium uredinicola* was present on the leaf surface.

Fusarium lateritium occurred in the phylloplane at senescence in the zone of foliar scars, before their suberisation, *Cytospora cincta* and *Eutypa lata* populations being present at the same time in high frequencies.

Various chemicals used to control diseases induced changes of phylloplane mycoflora composition, thus influencing negatively the saprophytic micro-flora playing a beneficial role in the biocenosis equilibrium.

Various chemicals used to control diseases after flowering and at fruit formation induced qualitative and quantitative alternations (Fig. 1). Within 24 hrs from treatment the phylloplane was occupied by species resistant to the fungicides applied, the species *Cladosporium herbarum*, *C. sphaerosperum* and *Aureobasidium pullulans* particularly prevailing.

After Bayleton applications *Trichoderma viride* and *Gonatobotrys simplex* persisted. A 0.2% rate of this compound enabled recovery of antagonistic microflora; two days later *Chaetomium* and *Acremonium* species have been isolated. The number of their colonies increased after 8 days, *Gliocladium roseum* and *Fusarium lateritium* being added. So Bayleton application did not disturb development of beneficial fungi in phylloplane in a considerable manner.

A similar action was also shown on the structure of saprophytic microflora following Captadin o.2% treatments. Fungi of *Cladosporium*, *Aureobasidium* and *Acremonium* genera have been recovered within a few hours from treatment, whereas after 2 days *Chaetomium* and *Trichothecium* species have also been isolated. Likewise, 8 days later some *Oospora* and *Acremonium* species were isolated.

Topsin 0.1% was the most active, showing a severe influence on epiphytic microflora, the total number of saprophytic fungi colonies with antagonistic action being very low during the first 4 days from application. Just after spraying only a few *Aureobasidium pullulans* colonies survived, among the beneficial saprophytic fungi in phylloplane. Two days later colonies of *Cladosporium* spp. and *Chaetomium* spp. have also been isolated whereas after 8 days populations of *Gliocladium roseum* and *Fusarium lateritium* were present in the phylloplane.

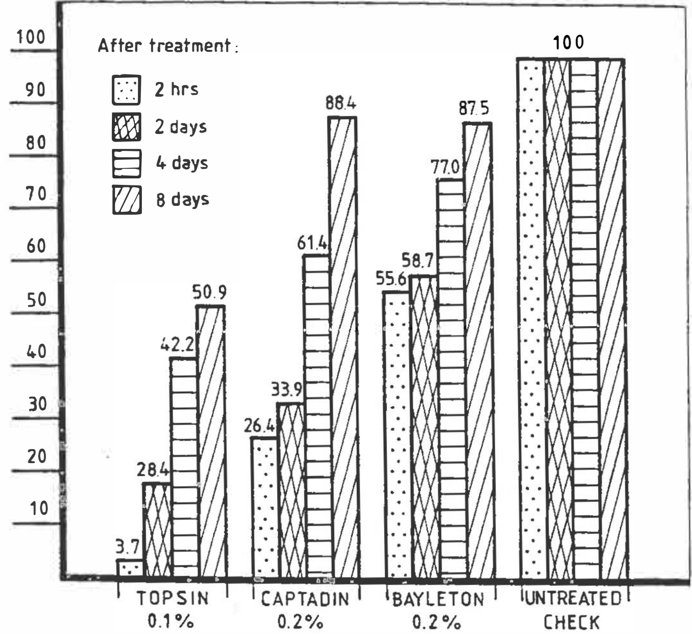

Fig. 1 Evolution of fungi with antagonistic action in the phylloplane in the variant treated vs. check (%)

Table 2. Evolution of the number of micromyceta colonies with antagonistic action in the apricot-trees phylloplane

Fungus	no. of colonies/ 19 cm^2					
	Bud scales		Floral	Leaves		Foliar
	winter	opening	organs	young	old	scars
Hyperparasitic						
Gonatobotrys spp.	0	0	12	0	0	16
Cladosporium spp.	8	12	22	20	17	0
Aphanocladium album	10	0	0	0	0	14
Acremonium alternatum	14	0	19	17	0	0
Oospora spp.	8	16	12	12	0	0
Antagonistic						
Trichothecium roseum	0	12	14	12	9	17
Trichoderma viride	0	0	5	0	0	0
Chaetomium globosum	0	8	8	11	0	0
Fusarium lateritium	0	8	12	14	18	22
Gliocladium roseum	0	12	0	0	0	0
Aureobasidium pullulans	18	15	17	21	16	26
Total colonies	58	83	121	107	60	95

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ASSESSMENT OF PESTICIDE SIDE EFFECTS ON BENEFICIAL MICROORGANISMS FROM THE PHYLLOPLANE

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Summary

The preservation of naturally occurring biological control agents as well as the introduction of antagonists against plant diseases require knowledge about the sensitivity of these organisms to pesticides. Subject of the investigations was the evaluation of laboratory methods for assessing pesticide effects on beneficial phyllosphere microorganisms. A technique for assessment of growth under pesticide influence in microplates by photometrical measurement of the optical density, being very sensitive, proved to be the most suitable method, appropriate for automatization and use in routine testing. This in vitro technique is discussed in comparison with data from experiments with plants.

Introduction

In entomology it is commonly accepted that pesticides are evaluated for their side effects on beneficial organisms like predators and parasites, in order to allow the application of biological control agents and to give room to naturally occurring biological control. Conversely, in the field of biological control of plant diseases this aspect, up to now, has been widely neglected, although a lot of knowledge has been accumulated on the ecological importance of microorganisms in the phyllosphere. Even if, to date, there are only few practicable approaches for an application of microbial biocontrol agents, under natural conditions microorganisms in the phyllosphere can exhibit considerable activity in antagonizing plant pathogens primarily by nutrient competition. As could be shown by FOKKEMA (1983), epiphytic yeasts can reduce the development of fungal diseases caused by necrotrophic pathogens by about 50 % if they are sufficiently established before the deposition of the pathogen.
The preservation of naturally occurring biological control agents as well as the successful introduction of antagonists against plant diseases require knowledge about the sensitivity of these organisms to pesticides. This is especially important in the phyllosphere as organisms in this habitat are particularly exposed to pesticides, being hit directly by pesticidal sprays and subsequently being confronted with the residues.

Field and greenhouse experiments

Some investigations on the effects of pesticides in field trials have been documented and reviewed by FOKKEMA (1988) and recently by DIK (1991). Own investigations on the influence of dichloflua-

nid (Euparen) and mancozeb (Dithane Ultra) showed that these fungicides strongly inhibit yeasts (Fig. 1) and prevent a recolonization for periods of several weeks (SMOLKA, 1992; SMOLKA & RUBACH, 1988). Experiments with plants and especially field experiments (Fig.2) are very time and material consuming, complex, and difficult to evaluate. Their results can easily be misinterpreted because of the complexity of influencing factors, and direct and indirect effects can't be differentiated. This, however, is a prerequisite if, for an evaluation of the potential risk field experiments are to be replaced by laboratory tests being appropriate only for the assessment of direct effects.

Laboratory methods for assessment of pesticide effects

Methods used up to now and described in the literature are summarized in Fig. 2. Subject of my investigations was the evaluation of suitable laboratory methods for assessing the effects of pesticides on beneficial microorganisms from the phylloplane. The aim was the elaboration of a standard method appropriate for routine testing. Main focus was on growth tests and the comparison with field data in order to evaluate their sensitivity and suitability for assessing effects occurring under natural conditions.

Material and methods

The methods were evaluated and optimized using several fungicides and insecticides. The experiments were conducted with a selection of epiphytic yeasts of the species *Sporobolomyces roseus* (H266, H272), *Cryptococcus albidus (H170, H177), C. laurentii (H274)* and *Rhodotorula minuta (H4, H35)* (isolated from tomato leaves), the bacteria *Bacillus* sp. (B9), and *Erwinia herbicola* (B129), all representing species very common in the phylloplane of many plants and acting as antagonists against fungal diseases. Two inhibition zone methods were used. Agar was mixed with the test organisms before plating for both methods. Then either filter paper disks soaked with pesticide suspension were plated onto the surface (paper disk plate method) or wells were cut into the agar and filled with the pesticide (agar well diffusion method). The third method used was the poison broth culture method, conducted in microplates. In this test, microbial growth under the influence of the fungicides was assessed by photometrical measurement of the optical density. Details of these methods have been recently described by SMOLKA (1992).

Results and discussion

Inhibition zone methods: The paper disk plate method was very insensitive and inappropriate to reveal effects observed in field experiments. For example, only the 10-fold of the recommended concentration of Dithane Ultra (mancozeb) produced inhibition zones, although in the glasshouse this fungicide caused a significant inhibition of yeasts for several weeks. The agar diffusion method with wells cut into the agar was slightly more sensitive. It showed, however, to be appropriate only for evaluation of the

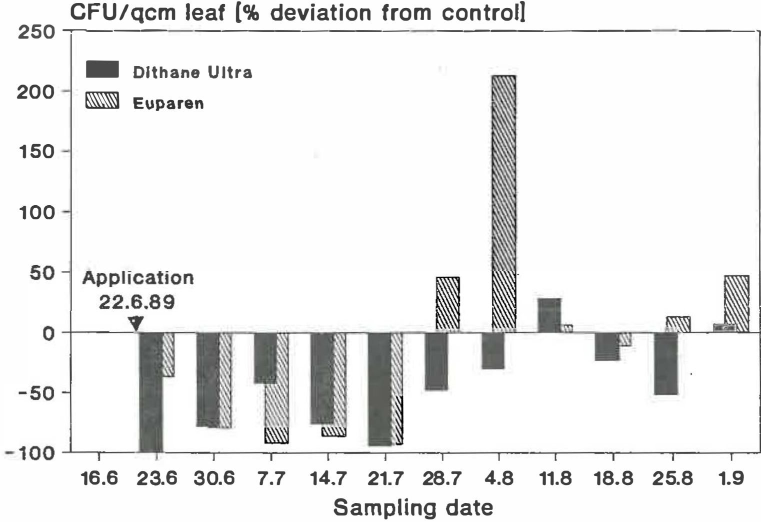

Fig. 1: Effects of Dithane Ultra (mancozeb) and Euparen (dichlofluanid) on yeasts in the phyllosphere of tomatoes under greenhouse conditions (CFU = colony forming units).

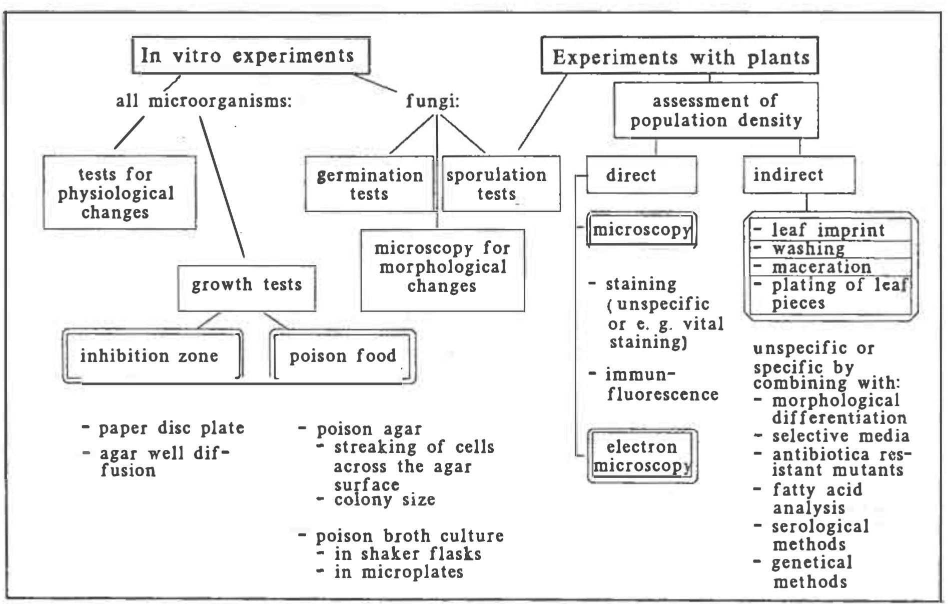

Fig. 2: Methods for assessment of pesticide effects on phyllosphere microorganisms.

effects of fungicide concentrations recommended for practical use and permitted only limited quantifications.

Tab. 1: Sensitivity of the agar well diffusion method and the poison broth culture method, conducted in microplates - comparison of the no-observed-effect-levels (NOEL) (yeast isolates H4, H35, H177).

Fungicide	NOEL in % of the recommended concentration	
	inhibition zone method (agar well diffusion)	poison food method (microplates)
Antracol (Propineb)	10	< 0,003 - 0,03
Dithane Ultra (Mancozeb)	2	< 0,003 - 0,01
Euparen (Dichlofluanid)	0,2 - 2	< 0,001 - 0,003
Polyram-Combi (Metiram)	10 - 100	< 0,003 - 0,03

Poison food technique in microplates: In contrast to the two agar diffusion methods the growth test in microplates could be shown as much more sensitive (at least by a factor of 100, Tab.1) and suitable for quantification of the activity of different pesticides and pesticide concentrations.
Fig.3 shows the effects of some fungicides on several isolates of yeast and bacterial species, common in the phylloplane. The strongest effects were measured after applying Euparen (dichlofluanid). The no-observed-effect-level (NOEL) of this substance was below a dilution of $1*10^{-5}$ of the concentration recommended for application on plants. The thiocarbamates Dithane Ultra (mancozeb) (Fig.3), Polyram Combi (metiram) and Antracol (propineb) caused an inhibition almost as strong as Euparen. The NOELs of Derosal (carbendazim) for most of the yeasts were between dilutions of 10^{-2}-$3*10^{-3}$ of the recommended concentration. It was, however, interesting that the most sensitive and the most insensitive isolate both belonged to the species *Sporobolomyces roseus* and this suggests the occurrence of resistant strains and is in accordance with other investigations (FOKKEMA, 1988). Bayleton spezial (triadimefon) and Ronilan (vinclozolin) were even less inhibitory to the test organisms with NOELs of about 10% of the recommended concentration and Previcur N (propamocarb) did not cause any significant growth inhibition of yeasts and bacteria.
These results demonstrate that the microplate method allows a clear differentiation between the effects of different pesticides and pesticide concentration as well as the sensitivity of different microbial species. As it can be automated, this technique can be used for testing a certain spectrum of microbial strains that reflects the spectrum of the microbial population in the phyllosphere.
This technique proved to be just as applicable for the evaluation of insecticides.

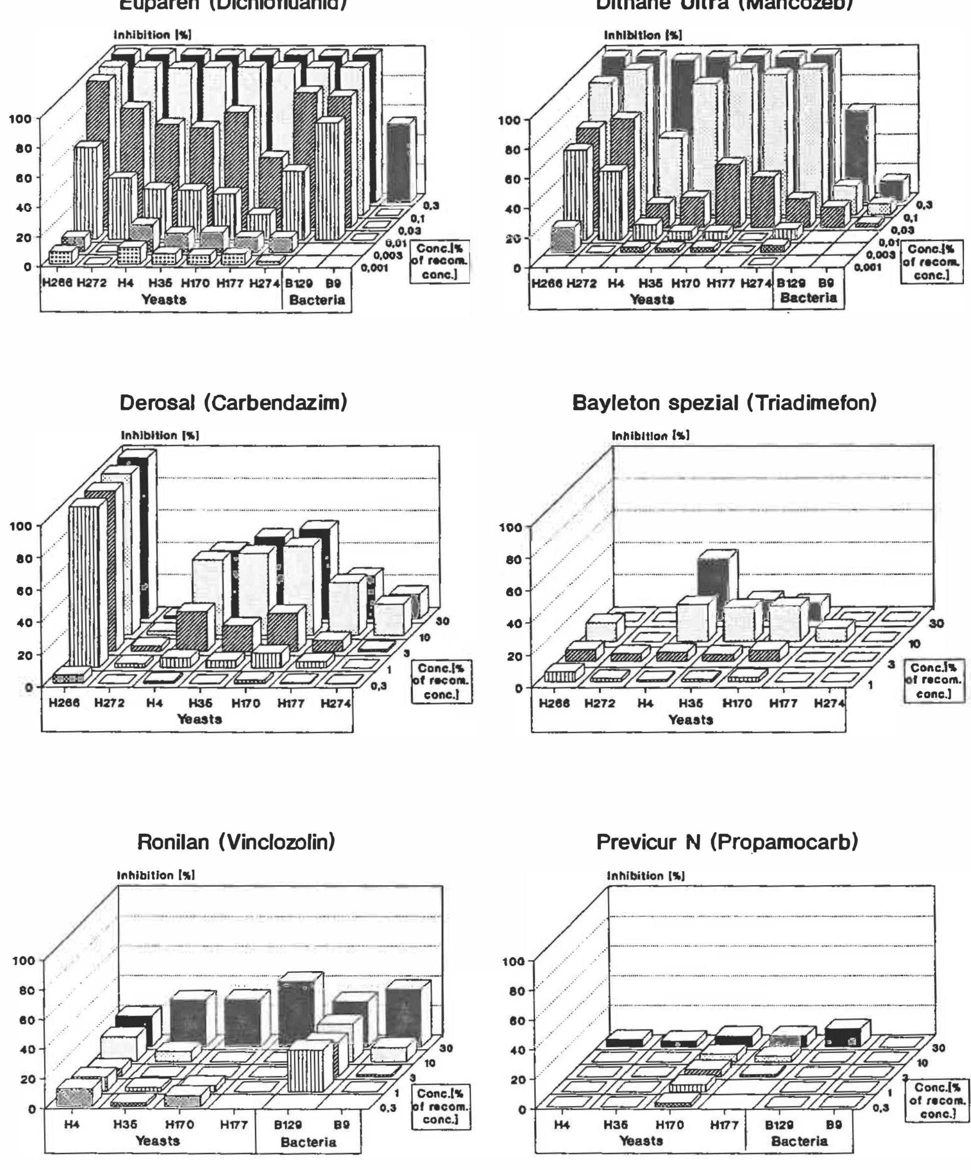

Fig. 3: Growth inhibition of phyllosphere microorganisms by fungicides assessed with the poison food technique in broth culture in microplates.

Unlike other in vitro methods this technique showed to be appropriate for revealing effects of fungicides observed in experiments with plants. For example, in vivo-effects of metiram could not be reproduced with normal concentrations by the inhibition zone methods but were revealed with the microplate method (SMOLKA, 1992). Effects of benzimidazoles observed in plant experiments but not found in vitro (FOKKEMA and DE NOOIJ, 1981) could be demonstrated with Derosal (carbendazim) using this method if non-resistant isolates were used. However, even with this technique the effects of benzimidazoles were by far not as strong as those of thiocarbamates. More information on the mechanism of action of the substances could be helpful in evaluating such results. For example, observations made by GROSS and KENNETH (1973) indicate that benzimidazoles inhibit spore production by *S. roseus* already in concentrations lower than necessary for growth inhibition. Under such circumstances it might be helpful to supplement the growth test by other methods such as a sporulation test, if pesticides are to be evaluated by in vitro tests.

However, if in the field, effects are found that cannot be revealed with laboratory methods, indirect effects like the disturbance of the natural balance between different species, for example, might be responsible. With respect to this aspect, the evaluation of results gained with in vitro methods demand information on the potential exposition of microorganisms on the phylloplane to pesticides. To date, I did not find any documentation on combined investigations on the development of the microbial population density after a pesticide application and the parallel degradation of pesticide residues or toxic breakdown products on the leaf surface. However, this is the most important question for an evaluation of the potential risk by in vitro methods and is a wide field for future research.

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INHIBITION OF PHYTOPATHOGENIC FUNGI BY PHYLLOSPHERE YEASTS IN AN *in-vitro* TEST

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Summary

In agar cultures some yeasts colonizing the phyllosphere were found to be antagonistic against some of phytopathogens from soil and/or leaf environment:
Hansenula holstii against *Pythium ultimum, Rhizoctonia solani, Gaeumannomyces graminis* and *Pseudocercosporella herpotrichoides. Candida foliorum* against *Rhizoctonia solani* and *Pseudocercosporella herpotrichoides, Candida bogoriensis* against *Pythium ultimum, Rhizoctonia solani, Sclerotinia sclerotiorum, Gaeumannomyces graminis, Verticillium albo-atrum* and *Septoria nodorum.*
On the other hand some yeasts stimulated growth and fructification of several phytopathogens as follows:
Tilletiopsis minor - Sclerotinia sclerotiorum, Candida javanica - Sclerotinia sclerotiorum, Candida bogoriensis - Phoma exiqua var. foveata.

Introduction

In view of a successful protection of the whole plant it seems to be necessary to treat with biological agent its root system and its above-ground part as well.
Biological treatment of roots should be followed by a further treatment of above-ground parts of plants by microbial agents against foliar diseases, e. g. by yeasts, which are able to colonize this environment and to regulate the occurrence of phytopathogens (BLAKEMAN and FOKKEMA 1982).
Both types of microbial agents, for the protection of roots as well as above-ground parts of plants, have to be able to be incorporated into biological preparations to be used in agricultural practice in usual ways.

Material and methods

In vitro inhibition of the pathogens by naturally occurring yeasts, characterized by 3 types of mode of action, was observed and evaluated according to the scale of SCHIEWE and MENDGEN (1992):

- The ability to induce demarcation zones
- The ability to reduce colony expansion by intermingling with the pathogen mycelium
- The over-growing the colony of pathogen

Phytopathogens used:
For the dual cultures of microorganisms, between pathogenic filamentous fungi and phyllosphere yeasts the following phytopathogens were chosen.

Oomycetes	:	*Pythium ultimum*
Ascomycetes	:	*Sclerotinia sclerotiorum*
		Gaeumannomyces graminis
Deuteromycetes	:	*Rhizoctonia solani*
		Verticillium albo-atrum
		Pseudocercosporella herpotrichoides
		Septoria nodorum
		Phoma exiqua var. foveata

Phyllosphere yeasts used:

Saccharomycetaceae	:	*Hansenula holstii*
Cryptococcaceae	:	*Candida foliorum*
		Candida bogoriensis
		Candida javanica
Sporobolomycetaceae	:	*Tilletiopsis minor*

Screening for antagonistic activity:
The antagonistic activity of the above mentioned yeasts was tested in vitro using 90 mm Petri dishes containing 10 ml of agar medium. Yeasts and filamentous fungi were screened on PDA agar, pH 6,4.
Agar plugs (diameter: 5 mm) taken from colonies of the phytopathogens were placed onto the plates 2 cm from the edge. Yeasts were streaked as a cell suspension on the opposite half of the plate.
Each dual culture (phytopathogen - yeast) had six replications. The plates were evaluated for antagonistic activity after 1 week and 5 weeks at 15°C.

Results and discussion

Isolates of yeasts reducing the development of phytopathogens by producing a demarcation zone or by intermingling with the pathogens mycelium or by over-growing the pathogens colonies were selected.
In vitro inhibition of the pathogens *P. ultimum, R. solani, G. graminis* and *P. herpotrichoides* by the yeast *Hansenula holstii* was characterized by the ability to induce demarcation zones and to reduce pathogen colony expansion. Especially a conspicuous lysis in the colony of soil-inhabitant *P. ultimum* followed by its disappearance from dual culture was remarkable. In the case of dual culture with *P. herpotrichoides, H. holstii* was able to spread actively into the colony of the phytopathogen.
The yeast *Candida foliorum* was able to inhibit by forming the demarcation zone only with *R. solani* but not with the other pathogens tested. *Candida bogoriensis* was the most successful antagonist which was able in 3 weeks to destroy the colony of *P.ultimum* completely and to reduce colony growth of *R. solani, S. sclerotiorum, G. graminis, V. albo-atrum* and *S. nodorum* causing a conspicuous lysis and inducting demarcation zones. An

exception to this was *Phoma exiqua var.foveata*, which on the contrary over-grew the colony of yeast with high pycnidia production. The fungus *S. sclerotiorum* over-grew the whole colony of *T. minor*, *C. javanica* and produced twice as much sclerotia as in the control.
In-vitro studies of interrelationships between some yeasts and phytopathogenic fungi coming from various environments showed their potential to inhibit or to stimulate each other. This knowledge should be verified in in-vivo trials.
Because some of fungi found on leaves frequently occur on seeds (LUND 1956) and some epiphytic species migrate from seeds to leaves (LEBEN 1961) it could be useful to screen couples of biological control agents together with the chosen yeasts against of the target phytopathogens.
Considering the control of phytopathogens it is necessary to take into account also the potential plant-microorganisms associations (STONE 1989) as part of the plant s physiological response to the interactions between plants and the beneficial yeast-like organisms.

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ESTABLISHMENT OF MICROBIAL INOCULA ON CROP RESIDUES

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Summary
The growth, interactions and cellulolysis adequacy indices of antagonists such as *Trichoderma harzianum* and *Gliocladium roseum* and straw-borne pathogens like *Fusarium culmorum, Rhizoctonia cerealis* and *Cephalosporium gramineum* were found to be markedly influenced by both water availability and temperature in *in vitro* studies on straw media. Such information in combination with growth studies on straw are critical to determining the competitive saprophytic capabilities of antagonists against pathogens.
The establishment and survival of antagonist fungi on crop residue was subsequently tested under field conditions. Studies were carried out with inoculants such as *T.harzianum* and *G.roseum* inoculated onto cereal straw in a wet and dry soil to determine their ability to survive, and their interactions with resident fungal populations, including *Fusarium culmorum* over periods of 3-6 months. This showed that establishment of inoculants on straw varied with soil wetness and with time. Such studies may help in determining the competitive abilities of biological control agents and their ecological competence.

Introduction
The successful establishment of microbial antagonists on crop residues is dependent on a number of factors. These include the nutritional status of the residue, the composition of the resident microbial community and environmental factors. Crop residue such as cereal straw has a very high C:N ratio (>100:1) and is often saprophytically colonised by pathogens as well as fungi characteristic of the phyllosphere and soil (Magan, 1988a). The ability of antagonist populations on such substrates to germinate, grow and compete successfully is predominantly determined by temperature, water availability, and pH of the residue and soil. This paper will highlight both *in vitro* and *in situ* experiments to determine the competitive ability of biological control agents such as *Gliocladium roseum* and *Trichoderma harzianum* against straw-borne diseases and survival on straw in soil.

Materials and Methods
Competitive saprophytic ability
Cellulolysis adequacy index (CAI) was determined by relating the ability of antagonists and pathogens to degrade filter paper and their growth on potato dextrose agar (PDA) at 22.5°C (Garrett, 1963). The mineral salt solutions and PDA were modified osmotically with NaCl to -0.3, 0.7 and 1.4MPa water potential (Lang, 1967). A high CAI (>1.0) is indicative of the ability to survive without additional nitrogen. Additional nitrogen could prolong survival of those fungi with a low CAI value.

Competition
Experiments were carried out at different water potentials and temperatures on a 2% straw agar using the numerical scoring system

developed by Magan & Lacey (1984). The score varied from 1 (intermingling), through 2/3 (mutual antagonism on contact or at a distance), to 4-5 (inhibition after contact or at a distance with the antagonist continuing to grow through the other fungus).

Germination and growth on straw and straw incorporated into soil

Either straw leaf sheaths or internodes of straw modified to different water potentials were inoculated with spores or mycelium inoculum of test fungi and germination, germ tube elongation and growth rate determined (Magan, 1988b;c). The inoculants *T.harzianum* and *G.roseum* were subsequently sprayed ($3x10^5$ spores ml^{-1}) in 0.1% peptone water at a rate of 0.2ml g^{-1} straw. This gave a final concentration of 6 x 10^4 fungal spores g^{-1} straw. Known subsamples of straw were placed in mesh bags and buried in covered soil plots which were regularly watered (wet soil; 0.1MPa) and a dry soil (1.4MPa). The patterns of colonisation was determined over 28 week period. Straw was washed in water, dried between two sheets of filter paper and either direct plated onto MEA and V8 juice agars modified to these same water levels; and also placed in diluent and serially diluted and plated onto selective media. The percentage colonisation of straw segments and the total populations of inoculants were monitored and compared in this experiment (Magan *et al.*, 1989). The effect of inoculants on other resident fungal populations was also determined.

Results

Laboratory studies

Table 1 shows that the CAI of both straw-borne pathogens and antagonists can markedly change with water potential. For example, *R.cerealis* needed additional nitrogen at -0.2MPa but not in a drier environment. By contrast, *G.roseum* had a high CAI at -0.2MPa but could barely survive at lower water potentials. *T.harzianum* had a consistently low CAI and for improved competitiveness would require additional nitrogen on cereal straw.

Table 1. Comparison of Cellulolysis adequacy index (CAI) of pathogens and antagonists based on relationship between degradation of cellulose filter paper and growth rate on PDA at 22.5°C.(Garret, 1963).

Fungus Water potential (-MPa)	R.c.	C.g.	P.h.	T.h.	G.r.
0.2	0.70	0.28	1.03	0.25	3.24
0.7	1.41	0.41	1.29	0.22	0.83
1.4	1.71	0.24	0.56	0.20	1.05

R.c., *Rhizoctonia cerealis*; C.g., *Cephalosporium graminis*; P.h., *Pseudocercosporella herpotrichoides*; T.h., *Trichoderma harzianum*; G.r., *Gliocladium roseum*.

Interactions between antagonists and pathogens were also found to be influenced by both temperature and water availability (Table 2). Often, dominance under one set of conditions was changed when temperature or water potential was altered.

Table 2. Effect of temperature and water potential on interactions between *T.harzianum* and straw-borne pathogens.

Fungus *T.harzianum*		*R.cerealis*	*C.graminis*	*P.herpotrichoides*
10°C	-0.7 MPa	4 - 0	2 - 2	2 - 2
	-2.8 MPa	3 - 3	1 - 1	2 - 2
15°C	-0.7 MPa	4 - 0	4 - 0	4 - 0
	-2.8 MPa	2 - 2	2 - 2	2 - 2

T.h. scores are given first

The ability of antagonists to effectively germinate and grow on the substrate and compete successfully with pathogens is also critical for establishment of biological control agents on substrates like straw. Table 3 shows that *Fusarium culmorum* is able to colonise unsterile leaf sheaths better than *T.harzianum* or *G.roseum*, although this is not evident in *in vitro* studies on agar.

Table 3. Comparison of spore germination and germ tube extension on unsterile straw leaf sheaths (L.sheaths) and that on straw agar modified osmotically (glycerol, SA+G) and matrically (PEG 6000, SA+PEG) after 24hr at 20°C.

		Spore germination (%)			Germ tube extension (um)		
Substrate		L.sheaths	SA+G	SA+PEG	L.sheaths	SA+G	SA+PEG
F.culmorum	-0.7MPa	72	100	74	412	>750	>750
	2.8	54	100	100	138	>500	479
T.harzianum	0.7	38	100	74	47	154	80
	2.8	17	97	19	10	47	9
G.roseum	0.7	55	97	100	67	144	163
	2.8	10	100	92	11	67	52

<u>Field studies</u>

The recovery of *T.harzianum*, applied at three different concentrations, and pathogenic isolates of *F.culmorum* from straw segments incorporated in soil for eight weeks is shown in Table 4. Generally, the highest treatment concentration of *T.harzianum* reduced the isolation of pathogenic isolates of *F.culmorum* from straw segments.

Table 4. Percentage straw segments yielding *T.harzianum* and *F.culmorum* during a eight week soil incorporation period.

	T.harzianum					*F.culmorum*				
Time	Control	Low	Medium	High	SED	Control	Low	Medium	High	SED
0	43.3	90.0	92.7	98.0	6.55	31.5	13.3	7.8	0	4.60
14d	22.7	46.7	51.3	75.3	10.18	40.8	36.2	36.5	17.5	9.29
28d	19.3	24.0	35.0	61.3	7.56	39.1	34.2	29.0	15.0	5.18
56d	26.7	34.7	44.7	76.7	8.14	42.1	37.5	39.2	23.0	10.45

Low, 3×10^4; Medium, 3×10^5; High, 3×10^7 spores g^{-1} straw.

The colonisation of straw by *T.harzianum* and *G.roseum* was influenced by soil moisture content. In a wet soil both inoculants were able to colonise the straw over a 28 week period. However, in dry soil the number of straw segments yielding these inoculants were markedly reduced (Table 5). By contrast, assessment of populations of these inoculants (CFUs) showed very little variation over time. This showed that the first analysis method may be a more accurate measure of assessing establishment on such substrates in soil. The presence of inoculants like *G.roseum* affected the colonisation of straw by other fungi, particularly in the dry soil. When *G.roseum* colonisation decreased (4-6 weeks) there was a significant increase in *Penicillium* spp. on the straw. *Gliocladium* spp. are known to produce antibiotics and this may be one reason for the inhibition or antagonism of other fungi colonising such a resource.

Table 5. Comparison of the isolation of *T.harzianum* and *G.roseum* from straw segments in a wet (-0.1MPa) and dry (-1.4MPa) soil over a 28 week period. Figures in parenthesis are the percentage segments yielding these fungi in the controls.

		Mean percentage straw segments				
Time (weeks)		0	4	8	12	28
G.roseum	wet	100 (0)	99 (11)	100 (1)	89 (1)	95 (0)
	dry	100 (0)	100 (15)	77 (5)	41 (1)	36 (0)
T.harzianum	wet	100 (0)	95 (27)	89 (3)	93 (0)	99 (36)
	dry	100 (0)	91 (16)	85 (1)	65 (1)	61 (7)

Discussion

Survival of pathogens on host residues in the face of competition from other microorganisms depends on their saprophytic capabilities. They can passively occupy the tissues, and survive vegetatively or they can actively occupy the tissue by extending their saprophytic activities into previously unoccupied tissues. Usually pathogens have an advantage as they already occupy the tissue and thus have a base to enable survival. By growing slowly and producing small amounts of extracellular enzymes they could reduce liberation of soluble nutrients from the substrate so discouraging competition. For displacement of such pathogens, or to prevent them expanding their base, antagonistic fungi must be able to effectively germinate and grow, possess suitable enzymes to utilize the available substrate (resource utilization), and be tolerant of adverse environmental conditions associated with the resource. The competitive saprophytic ability of antagonists needs to be determined to enable effective primary or secondary resource capture. This study has shown that by using appropriate ecological criteria, *in vitro* and laboratory scale experiments it is possible to screen for inoculants which may be able to successfully and actively compete for such substrates under naturally variable environmental conditions and control pathogens which survive on crop residue.

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DROUGHT TOLERANCE AS A MAJOR SELECTION CRITERIUM FOR ANTAGONISTS OF *BOTRYTIS* SPP.

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Summary

Saprophytic antagonists can be applied to suppress sporulation of *Botrytis* spp. and other necrotrophs on necrotic tissue. In a screening based on bio-assays on dead onion leaf tissue in moist chambers, potential antagonists were selected which suppressed sporulation of *Botrytis aclada* almost completely. Besides antagonism, the ecological competence of candidate antagonists must be tested under controlled conditions. One of the main factors limiting the performance of antagonists in above-ground necrotic tissue are the extreme changes of the water content of the substrate.

In bio-assays, wetness periods were interrupted 13 h, 39 h, or 63 h after application of the antagonists with dry periods of 6 h. Antagonists belonging to *Alternaria* spp., *Ulocladium* spp., *Chaetomium globosum* and *Aureobasidium pullulans* were still efficient after dry periods. *Gliocladium* spp., *Trichoderma harzianum*, *Penicillium* spp., *Arthrinium* spp. and *Sesquicillium candelabrum* were sensitive to dry periods in the early stage of colonization of the substrate and did not suppress the sporulation of *Botrytis aclada*.

Introduction

The suppression of sporulation on necrotic leaf tissue and on plant remains is a feasible strategy for the biological control of *Botrytis* spp. and other necrotrophs (Köhl et al., 1992b; Köhl et al., this volume). Antagonists competing with *Botrytis* spp. in such substrates must be able to withstand the characteristic fluctuations of the water content. For the selection of drought tolerance, two criteria can be defined: (1) Spore germination and mycelial growth of antagonists and their antagonistic efficiency in dead tissue at low water potentials between -3 MPa and -7 MPa, and (2) survival of the antagonists in the dry substrate and rapid growth and high antagonistic efficiency after dry periods. Antagonists differ in their ability to grow at low water potentials. Amongst antagonists suppressing ascocarp formation of *Pyrenophora tritici-repentis* on straw, Pfender et al. (1991) found only a few antagonists such as *Acremonium terricola*, *Epicoccum nigrum*, *Myrothecium roridum* and *Stachybotrys* sp. reducing the colonization of straw by *Pyrenophora tritici-repentis* even at -7 MPa. In bio-assays with dead onion leaf segments, *Gliocladium roseum* and *Trichoderma viride* suppressed the sporulation of *B. aclada* almost equally on moist leaves at -1 MPa. At -6.6 MPa the more drought tolerant *G. roseum* was still efficient, whereas *T. viride* failed to suppress the sporulation of the pathogen at such a low water potential (Köhl et al., 1992a).

The objective of our study was the selection of antagonists effective after interrupted wetness periods.

Selection of antagonistic saprophytes

In preliminary bio-assays based on dead onion leaves precolonized by *B. aclada*, antagonists had been selected which suppress the sporulation of *B. aclada* under continuous wet conditions in moist chambers. Selected antagonists such as *Alternaria alternata*, *A. tenuissima*, *Arthrinium montagnei*, *Ar. phaeospermum*, *Chaetomium globosum*, *Ulocladium atrum* and *U. chartarum* originated from dead onion leaf tips. Several isolates of *Trichoderma harzianum*, *T. viride*, *Gliocladium catenulatum* and *G. roseum* were also found effective in suppression of sporulation of *B. aclada* under continuous wet conditions (Fokkema et al., 1992).

Water dynamics in necrotic onion leaves

The water dynamics in dead onion leaf tips were monitored in an onion field in summer 1992. Therefore, sensors were developed at our institute measuring the capacity between two pins inserted into the dead leaf tissue. The capacity of the dead tissue decreases with increasing water content. The temperature inside dead onion leaf tips, the relative humidity of the air and air temperature in the crop were also recorded. A first analysis of the data indicate that the wetness periods in leaves after dew nights or rain showers were mostly interrupted rapidly by dry periods (Fig. 1). Water contents resulting in water potentials of approximately -3 MPa to -7 MPa were recorded only for short periods, mostly shorter than 30 min., during the drying process of the dead leaf tissue. Also on misty or rainy days, the wetness periods frequently were interrupted for short periods. It was concluded from these results that for antagonists applied to necrotic tissue above the ground, their adaption to interrupted wetness periods is a main factor determining their ecological competence. On the other hand, growth and antagonistic activity at water potentials between -3 MPa and -7 MPa, occurring in such substrates only for short periods during the drying process, may be less important.

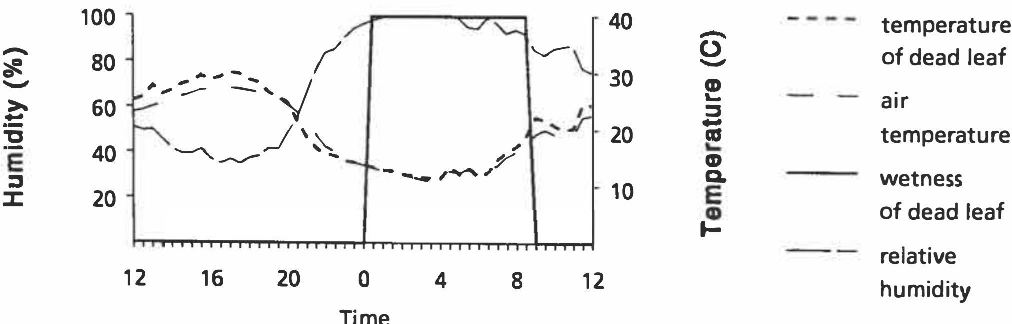

Fig. 1 Wetness period and temperature of dead onion leaf tips and micro-climate in an onion crop (data from 05 / 08 August 1992)

Bio assay with interrupted wetness periods

Material and methods

A bio-assay was developed to study interactions between *Botrytis* spp. and antagonists in necrotic onion leaf tissue after interrupted wetness periods. Green, healthy leaves of onions var. Hyton were dried at 60°C, sterilized by gamma-radiation and washed thoroughly three times to remove soluble nutrients. Leaf segments (3 cm long) were placed into moist chambers. The dead leaf segments were sprayed with conidial suspensions (1×10^5 ml^{-1}) of *B. aclada* and were incubated for 24 h at 18°C. Thereafter, conidial suspension of antagonists (1×10^6 ml^{-1}) or water as control treatment were sprayed on the leaf segments. At day 1, 2 or 3 after application of the antagonists, after an incubation period in moist chambers of 13 h, 39 h, or 63 h, leaves were transferred onto dry sterile filter paper and were placed into a laminar flow cabinet under sterile conditions, where the leaves dried within approximately 1 h. Leaves were kept dry for 6 h and rewetted again. In additional treatments, leaves were dried, kept dry for 6 h and rewetted again on day 1 and 2 or on day 1, 2 and 3 after the application of antagonists. Rewetted leaves were incubated in moist chambers. The leaf surface covered with conidiophores of *B. aclada* was estimated 8 d after application of *B. aclada*.

Results and discussion

The results of one of the bio-assays are shown in Fig. 2 as an example. In this bio-assay, the sporulation of *B. aclada* was abundant after incubation under continuous wet conditions. The sporulation was slightly reduced after one dry period, but significantly lower when dry periods had been repeated at two or three subsequent days. *G. roseum* suppressed the sporulation of *B. aclada* completely under continuous wet conditions, but failed to control the pathogen when the leaf wetness had been interrupted at the first day after application of the antagonist. When leaves were dried only at the second or the third day after application, *G. roseum* suppressed the sporulation of *B. aclada*, but to a lesser extend than under continuous wet conditions. These results indicate that *G. roseum* is sensitive to dry conditions during the early stage of leaf colonization. The antagonistic efficiency of *Alternaria alternata* and *Chaetomium globosum* was never reduced after interrupted wetness periods. Also *Arthrinium montagnei* was relatively resistant to dry conditions.

In general, antagonists belonging to *Alternaria* spp., *Ulocladium* spp., *Chaetomium globosum* and *Aureobasidium pullulans* were efficient in suppression of sporulation of *B. aclada* also after interrupted wetness periods. *Gliocladium* spp., *Trichoderma harzianum*, *Penicillium* spp. and *Sesquicillium candelabrum* were sensitive to dry periods. Especially in the early stage of colonization of the substrate they did not efficiently suppress the sporulation of *B. aclada*, which may indicate that germinating spores of these fungi are not drought resistant. Diem (1971) found that the survival of germinating spores during periods of low humidity differs significantly between fungal species.

Antagonists selected in bio-assays with interrupted wetness periods will now be applied in field experiments to test their ability to colonize necrotic leaf tissue, to survive in the substrate and to suppress sporulation of *Botrytis* spp. under field conditions.

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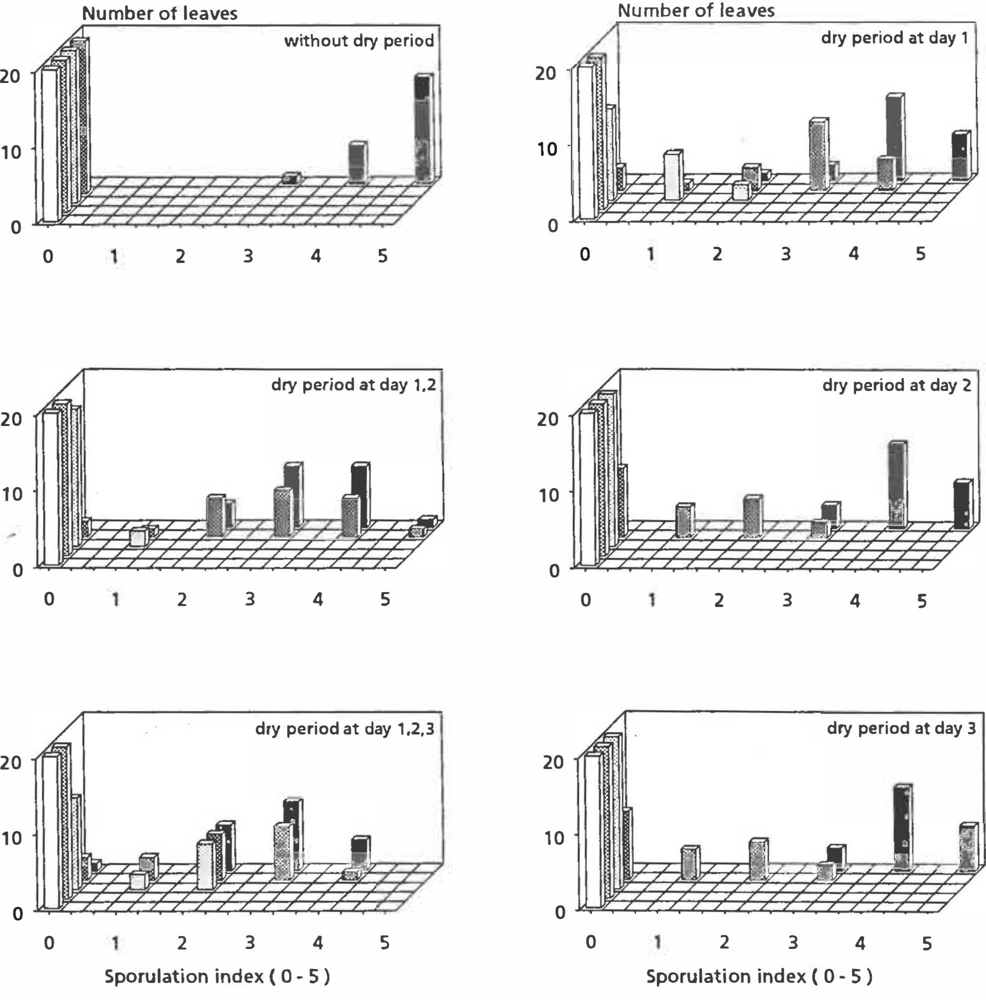

Fig. 2 Effect of dry periods on the suppression of sporulation of *Botrytis aclada* by *Alternaria alternata* (▭), *Chaetomium globosum* (▭), *Arthrinium montagnei* (▭) and *Gliocladium roseum* (▭) compared to a water treated control (▭) in a bio-assay on dead onion leaf segments. Conidia of *B. aclada* were sprayed on all leaves 24 h prior to antagonist treatment (Sporulation index: 0 = no sporulation of *B. aclada*, 1 = 1-5% leaf surface covered with conidiophores, 2 = 6-25%, 3 = 26-50%, 4 = 51-75%, 5 = 76-100%)

DNA FINGERPRINTING OF A *TRICHODERMA* ISOLATE BY THE RAPD PROCEDURE.

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Summary

The Random Amplified Polymorphic DNA (RAPD) method was carried out with DNA of 47 *Trichoderma* isolates using arbitrary primers of ten-bases. This procedure allowed us to distinguish among isolates from three aggregate groups of the *Trichoderma* genus. Some of the isolates which were identified as *T. harzianum* had similar RAPD patterns with 30 primers. Among the isolates of the *T. viride* group only a few had similar patterns, whereas isolates of the *T. hamatum* group were not similar to one another. The fingerprinting of the biocontrol agent T-39 as determined with a set of five primers was found to be unique. The method which is fast and reproducible can be very useful for identification of biocontrol agents of this group.

Introduction

Isolates of the genus *Trichoderma* have been reported as effective biocontrol agents against several plant pathogens (Chet, 1987). The genus *Trichoderma* was characterized by Rifai (1969) according to descriptions of colony growth, conidiophore, and observations on deviations from the typical conidiophore, phialide and conidium morphology. On the basis of these observations, the strains were assigned to nine species aggregates. This classification was highly artificial and significant variations remained to be defined in each of these groups. A partial revision of the genus was done by Domsch et al.(1980) and later by Bissett (1984), in order to find a useful system for differentiating among phenotypically alike species in the genetically heterogenous species aggregates.

The use of comparison among isolates according to their isoenzyme patterns has been suggested in the past (Zamir & Chet, 1985), but this comparison may only be useful under standardized conditions, as it is well known that extracellular enzymes are highly substrate dependent. With the growing interest in the use of *Trichoderma* species as biocontrol agents, it is essential to have a complementary system for identification.

We demonstrate here the use of the RAPD technique as a method for identification (Williams et al., 1990). This method, which uses the PCR (Polymerase Chain Reaction) technique, does not depend on DNA sequence information and can generate fingerprinting from only a small amount of DNA. The DNA amplification was carried out with arbitrary primers as short as 10 nucleotides. These chosen primers produced detailed and relatively complex profiles, which enabled us to detect DNA polymorphism among the *Trichoderma* isolates and to distinguish between the biocontrol agent T-39 and others.

Methods

Table 1. Isolates of *Trichoderma*

Trichoderma species & origin	Strains
T. harzianum	
Israel	(39[1],28[1],35[2],132[1],33[2],315[2],y[2])
Germany	(011[3],020[3],045[3],087[3],088[3], 120[3], 124[3],150[3])
Sweden	(055[3],058[3])
U.S.A	(94[2],96[2])
Norway	(Th107[5])
Switzerland	(168[3],170[3])
Iran	(000[3])
T. viride	
Germany	(002[3],003[3],004[3],009[3],048[3],122[3],141[3],155[3],190[3],082[3],085[3],109[3])
France	(99[1])
Canada	(LP55[4])
Norway	(300[3],304[3],Tv1[5])
Austria	(294[3],285[3])
T. hamatum	
U.S.A	(44[2])
Germany	(025[3],047[3],049[3])
Switzerland	(166[3])

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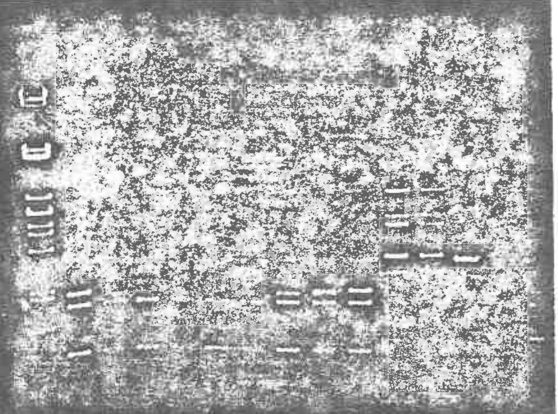
Trichoderma genomic DNA preparation: A modification of the preparation method suggested by Raeder & Broda (1985) was employed.
Primers: Random primers each of 10 nucleotides, from Operon Technologies were used.
PCR amplification: Amplification reactions were performed in 25 ul containing 1 unit of Taq polymerase, 2.5 ul of buffer x10, 2 ul of dNTP (1.25 mM), 1 ul of $MgCl_2$ (10mM), 0.5 ul of (10uM) primer and 25 ng of DNA. The reaction mixture was overlaid with mineral oil and run for one cycle through the following temperature profile: 94C for seven minutes to denaturate, 35C for one minute for low stringency annealing of the primer and 72C for two minutes for extension. Forty additional cycles were run according to the following protocol: 95C for one minute, 35C for one minute and 72C for two minutes. At the end of these cycles, an additional run at 72C for five minutes was conducted. The amplification was performed in a Hybaid Thermal Cycler and the

products were analyzed by electrophoresis in 1.2% agarose gels and detected by staining with ethidium bromide.

Results and Discussion

Genomic DNA of 47 *Trichoderma* isolates from 3 aggregate groups were each amplified by 3 different primers. Results obtained showed that within the *T. harzianum* group 10 out of 23 isolates produced similar RAPD patterns. These isolates, including the biocontrol agent T-39, were originated from various geographic locations. All the other isolates which classically were identified as *T. harzianum* produced different patterns. In order to generate distinguishable RAPD patterns for the biocontrol agent, additional 35 primers were screened. As illustrated in Fig 1, one of the primers could differentiate among 4 isolates including T-39 and the other 6 isolates. Additional 3 primers were necessary in order to distinguish between T-39 and theses 3 isolates. The uniformity in the DNA fingerprinting of 10 isolates within the *T. harzianum* group suggested that they may belong to a new subgroup. The isolates of the *T. viride* group were less uniform on the basis of the RAPD patterns (Fig 2). Amplification products with one of the primers resulted in similar patterns with only 3 couples of isolates. The isolates of the *T. hamatum* group produced different patterns for each of the isolates with the 3 primers that were examined. The classification of *Trichoderma* species and especially the ability to distinguish one strain from another is a very important issue in research of biological control. The method we present in this work suggests that it is possible to identify a specific strain on the basis of it's DNA fingerprinting. The ability to identify a specific isolate is important, especially once a biocontrol agent is sprayed in the field and there is a need to monitor its population. The methods used traditionally to monitor the survival of a biocontrol agent under field conditions only distinguish between *Trichoderma* isolates and other fungi but there is no way to identify a specific isolate. The relationship among isolates that have similar RAPD patterns is not clear yet, however, this technique may be useful for more detailed classification of the *Trichoderma* genus.

Figure 1: Gel electrophoresis of DNA amplification products generated with *T. harzianum* isolates. PCR was carried out with primer No.1. the letters a-n represent isolates: 39 ,000 ,020 ,045, 055, 058 ,087 ,168, 170, 94 ,96 ,y ,088, 011 respectively. M=size standards obtained by digesting lambda DNA with PstI.



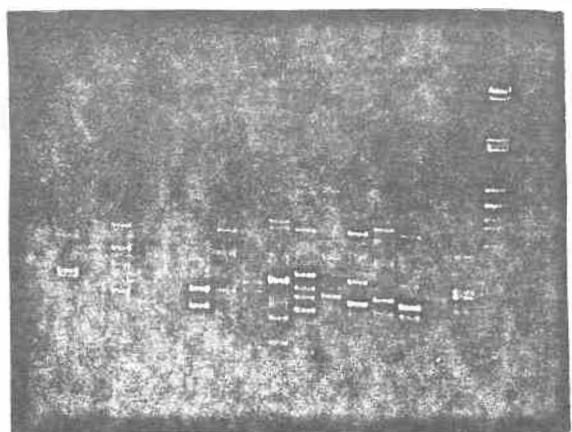


Figure 2: DNA amplification products of *T. viride* isolates using primer No. 3. Letters a-p represent isolates: 304, 300, 082, 085, 122, 285, 141 ,048, 155, 294, 190, 009, 003, 002 ,lp55 ,T-39 respectively. M=size standards obtained by digesting lambda DNA with PstI.

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DISCUSSION: ECOLOGY

NYCKLE J. FOKKEMA

It was generally acknowledged that the paper by Dik & Clayton indicated in what direction our knowlege should develop in order to understand the behaviour of introduced antagonists. Prior to any attempt to explain changes in population density of the micro-organisms, we have to address the question whether we have estimated its density correctly. This might be possible for individual leaves but if we like to kwow the population density of an antagonist in a crop in order to predict its role in crop protection, which may be different from leaf protection, we should be sure that our sampling technique is adequate for obtaining information on a field scale. It has been made clear that the distribution of phyllosphere bacteria in a field may not follow a random pattern. In such instances random sampling provides wrong information and therefore modified sampling methods should be developed.
In addition it is known that on a reduced scale of e.g. one square meter bacterial populations of individual leaves are generally lognormally distributed, whereas yeasts and hyphal fungi on the same leaves follow a normal distribution (see proceedings of phyllosphere symposia, 1986 and 1991).

Although there are very few examples of simulation models for antagonist populations yet, it is clear that they are powerful tools in estimating the population dynamics of antagonists under different environmental conditions. When these models are linked to models simulating the pathogen population and disease development, the performance of BCA's in the field may be predicted. Knowledge of circumstances in which biocontrol is likely to fail is essential in integrated control where chemicals may be applied if these circumstances occur.

A sensitive in vitro method has been developed for detecting adverse effects of pesticides on standard set of known naturally occurring antagonistic yeasts and bacteria. Use of pesticides with a negative effect on benefical micro-organisms should be restricted in situations where micro-organisms are likely to play a significant disease suppressing role. In contrast to our knowledge of the phyllosphere microflora of field-grown plants, little is known of naturally occurring micro-organisms on leaves of glasshouse crops. The densities of yeasts on indoor-grown tomato leaves mentioned by Dr Smolka are higher than previously expected.

Saprophytic colonization of dead plant material is determined by temperature, water availability and pH. Because of the very high C:N ratio, nitrogen amendments may favour colonization. Quantitative determinations of the saprophytic populations are difficult, because methods based on colony-forming units overestimate heavily sporulating fungi. In bio-assays for screening potential antagonistic colonizers of dead tissue, visual assessment seems to be sufficient. The ability to withstand rapid changes between wet and dry periods is a crucial selection criterion for antagonists operating in dead plant material.

Mode of action of BCA's and biorational control

MODE OF ACTION OF BCA AND BIORATIONAL CONTROL

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Introduction

It is not the purpose of this introduction to provide a complete review of the modes of action of biological control agents (BCA's). Several recent reviews have been written with coverage of this topic and these should be referred to for further information (Cook & Baker, 1983; Blakeman, 1988; Fravel, 1988; Whipps *et al.*, 1988; Wilson & Wisniewski, 1989; Andrews, 1992). Instead the intention is to introduce the topic area with an overview of the subject matter together with suggestions for possible future research in this area. In doing this it is important to understand the basic underlying principles and mechanisms of action by which the BCA operates in order to perfect systems for biological control of foliar and postharvest diseases. Once this mode of action has been worked out then it may be possible to optimise the BCA directly as a means of pathogen control or more indirectly to resort to manipulation of the mechanism of antagonism without using the antagonist itself for biocontrol purposes (e.g. spraying with antibiotics; interference with enzyme production; induction of host defence mechanisms by metabolites and subcellular components etc.). This latter strategy is biorational control. Both the use of the BCA itself and its exploitation by biorational control require an in depth knowledge of the interaction between pathogen, BCA and the microenvironment associated with the plant surface. These interactions, the elucidation of the modes of action involved and their potential exploitation for biocontrol purposes are dealt with in this section.

Mode of Action

For biocontrol purposes the BCA is an antagonist to the pathogen and in many cases will act via one or more of the modes of action outlined in Table 1. In general, the mode of action may be given as antibiosis, competition for nutrients or space, hyperparasitism or induced resistance but more often than not there is little information about the actual mechanism of action at the biochemical and ecophysiological levels in the *in situ* situation on the plant surface. It is these details of the mechanisms of action that need to be clarified in order for the mode of action to be exploited for biocontrol purposes.

Studies directed at antibiosis as the mode of antagonism invariably prove to be problematic. In many cases this is because the antibiotic substances have not been isolated, purified and identified and this almost certainly leads to confusion since it is not clear what the comparative observations might be in the absence as well as the presence of the antibiotic. This may or may not be the case *in vitro* where it is easier to demonstrate antibiosis but in the *in planta* and finally *in situ* situations one must be able to demonstrate beyond any doubt that antibiosis is operative and this has often proved difficult to substantiate in the past. There have been reports where *in vitro* studies have suggested that antibiosis was the mode of antagonism but then *in planta* experiments indicated colonisation of the plant surface involving competition for nutrients and space was the mode of antagonism (Utkhede & Rahe, 1983). Careful observation therefore must be made when moving from *in vitro* to *in*

planta and then on to *in situ* situations. Finally, if antibiosis is the mode of antagonism *in situ* then the details of the mechanism of action must be clarified in order that the biocontrol can be improved, manipulated and perfected. Once the details are fully elucidated it may even be possible to use the antibiotic itself as a biorational control.

Table 1. Modes of action of BCA and examples of parameters needed to perfect biocontrol or biorational control.

Mode of Action	Factors and Parameters involved	Details needed to improve or perfect the system
Antibiosis	Antibiotics produced by bacterial and fungal antagonists (e.g. bacilysin, agrocin, furanone)	Purification, identification of antibiotic - action *in planta* as well as *in vitro* clarification of mechanism of action
Competition for nutrients	Natural exudates from leaf surface and fruits; Release of nutrients on lysis of plant cells	Identification of specific nutrients; Use of analogues Biorational control
Competition for space	Topography of microbial and plant surface; attachment sites	Identification of plant waxes and cell wall components of antagonist and pathogen involved; Avirulent strains
Hyper-parasitism	Chitinases, ß-,1,3-glucanases, lytic enzymes	Regulation of synthesis, release and substrate specificity
Induced Resistance	Hypersensitive response, elicitors, phytoalexins, chitinases, ß-1,3-glucanases	Modulation of response to raise level of plant resistance; Avirulent strains

Competition for nutrients and space are no less easy to verify *in situ* and in many situations these have been implicated indirectly where it has been shown that antibiosis is not the mode of action. Work is required to determine the nature and levels of the nutrients involved especially in relation to the plant surface (leaf, flower, fruit, etc.). Particular emphasis is required to determine the importance of individual nutrients to BCA and pathogen in order to promote the BCA and repress the pathogen. It may well be that only a slight shift in nutrient balance is needed to allow the BCA supremacy over the pathogen. The use of nutrient analogues could prove useful here and could even be successful in biorational control once the component nutrients have been identified and their order of importance established. Competition for space has

been implicated for several BCA's and the more convincing work has been that using avirulent mutants. When competition for space is the mode of action it is almost always necessary to occupy the plant surface with the BCA prior to invasion by the pathogen in order for biocontrol to be effective. This, together with the use of avirulent strains, suggests specific sites of attachment but little is known concerning the nature of such sites. More work is necessary in order to identify these molecules and indications are that surface moieties such as glycoproteins and lipopolysaccharides are involved (Romantschuk, 1992). It may also be possible to exploit the mechanisms of this phenomenon for biorational control.

Hyperparasitism of fungal plant pathogens is in many cases associated with the production of lytic enzymes by the BCA especially chitinases and ß-1,3-glucanases since chitin and ß-1,3-glucan polymers are major components of the fungal cell wall. Again the enhancement of production and release of these enzymes by the BCA or the use of the enzymes themselves for biorational control could be used to suppress the pathogen. Induced resistance in the plant is a complex phenomenon and can be invoked by several factors (Table 1). Recently a protein (termed Harpin) associated with the cell envelope of the bacterial plant pathogen Erwinia amylovora has been implicated in the hypersensitive response. The gene for this protein has been identified and mutated with the use of the transposon Tn5 tac1 and non-pathogenic avirulent strains of E. amylovora engineered (Wei *et al.*, 1992). This work offers future possibilities and begins to unravel the complexities of pathogenicity and induced resistance for disease control. Induced resistance can also be provided by elicitors of plant and microbial origin. In the latter situation both pathogen and BCA extracts have been used to provide induced resistance. The plant itself may produce phytoalexins, chitinases and ß-1,3-glucanases, all of which have been indicated as factors involved in induced resistance (Cook, 1981; Sequeira, 1984).

Future research

It is obvious that there is insufficient detailed information available at present concerning the mechanism of action of BCA's against microbial plant pathogens in situ. The interaction of the BCA, pathogen and host plant in the microenvironment of the infection court requires further study but the methods available to carry out such ecophysiological monitoring have been limited in the past. Even now such work is limited but new methodologies are being developed that will allow a greater understanding of these systems. The use of low temperature scanning electron microscopy (LTSEM) is proving useful to visualise the pathogen and BCA in situ on the plant surface. Some interesting observations can be made concerning the spatial relationship and activity (developmental stage) of both pathogen and BCA and in addition the relative numbers of each for biocontrol purposes can be estimated. Such work at the moment is tedious and time-consuming and new approaches are required to allow easier and more rapid monitoring of this interactive ecophysiology. The use of vital stains such as Calcofluor M2R (an optical brightener) should prove useful in this respect (Gull & Trinci, 1974; Edwards & Seddon, 1992). It would then be possible to carry out observations of the mechanism of action of the BCA in the microenvironment where it is to be introduced rather than to resort to washing and retrieval methods for microorganisms or the use of in vitro studies and then extrapolation of the findings to the in planta situation which is never satisfactory.

So far ideas have been focused around the modes of action already established (Table 1), but there are other modes and mechanisms of action as yet unexplored. It has been stated that "there remain many uncharted mechanisms that have evolved and are functioning undetected in the natural world" - Baker

(1987). Chemotaxis is one such mechanism that deserves further investigation. Many of the microbial plant pathogens and, indeed, the BCA's that are antagonistic to them, are motile and can respond to chemical (nutrient) gradients. Interference with and exploitation of this phenomenon may well prove useful for future biocontrol studies (Klopmeyer & Reis, 1987). Touch sensing is yet another mechanism which is only poorly understood but which may have strong potential for development of biocontrol mechanisms. Touch sensing involves the pathogen being able to identify and measure the topography of the plant surface. Recent work with the barley brown rust has shown that this pathogen effectively measures the height, depth and distances across leaf cells in order to locate guard cells and penetrate the leaf (Read *et al.*, 1992). The possibility therefore exists of disrupting this mechanism and one way of doing this may be to breed plants with different surface morphology. Finally one novel method with future potential for biocontrol is being pursued at Aberdeen. It has been found that bacteria which have had their cell wall components removed can sometimes regenerate in this cell wall-less state (L-form bacteria) and, more importantly, can associate intracellularly with plant cells (Paton & Innes, 1991). Studies indicate that if the parent bacterium is a pathogen then plants which are associated with these derived L-forms are not infected and moreover are protected from the pathogen (Amijee *et al.*, 1992). If this proves to be a general phenomena then this would indeed be a novel method of biocontrol.

Future research must not be blinkered but must exploit and integrate the whole range of modes and mechanisms of action of BCA's if biocontrol is ever to become a practical option for agricultural purposes.

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POSSIBLE MODES OF ACTION OF YEAST ANTAGONISTS OF POSTHARVEST DISEASES

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Summary

The mode of action of many postharvest biocontrol agents is poorly understood. The lack of knowledge is attributed to a limited understanding of the interactions between the host, the pathogen and the antagonist. Several mechanisms of action have been suggested which include: antibiosis, competition for nutrients and space, induction of host resistance processes and direct interactions between the antagonist and the pathogen.

Introduction

Our knowledge on the mode of action of most postharvest biocontrol agents is meager. It has mostly followed concepts of mode of action based on traditional screening procedures. These would favor the selection of antagonists which act by direct interactions or by the production and secretion to the growth medium of substances toxic to the pathogens (antibiotics). Screening programs based on this concept are likely to disregard potential antagonists among the epiphytic microflora with other modes of action.
The lack of thorough knowledge on the modes of action of postharvest biocontrol agents, other than antibiosis, may be attributed to our limited understanding of the interactions taking place between the host, the pathogen and the antagonist at the site of infection. Yet, information on the mechanisms of antagonism is crucial for developing successful postharvest biocontrol strategies. Such information is essential for (a) optimization of the method and timing of application of the antagonist, (b) developing appropriate formulations to enhance antagonist efficacy, (c) developing a rational for selecting more effective antagonists and (d) the registration of biocontrol agents for commercial use.

Results and discussion

The following modes of action have been considered:

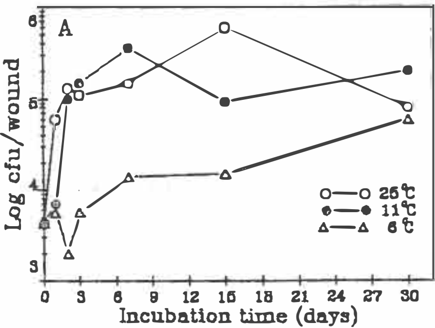
1. Antibiosis

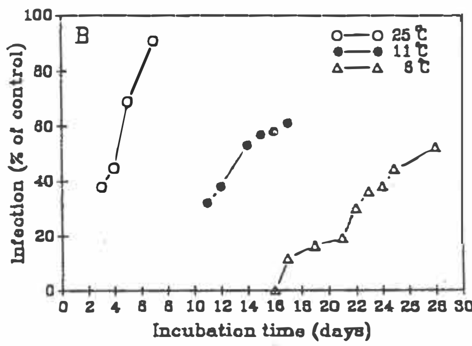
Secretion of antibiotic substances is a common phenomenon in nature. A number of bacterial antagonists have been reported to produce antibiotics *in vitro*. These may have a role in protecting commodities against diseases after harvest. The bacterium *Bacillus subtilis* and *Pseudomonas cepacia* has been reported to produce potent antibiotic substances, iturin and pyrrolnitrin, respectevely, (Gueldner et al., 1988; Roitman et al., 1990). A crucial question is whether production of the pathogen-inhibitory substance in culture is indicative of its involvement on the plant. The role of pyrrolnitrin in the mode of action of the bacterium *Pseudomonas cepacia* was recently questioned by Smilanick and Denis-Arrue (1992) since a pyrrolnitrin-resistance isolate of the pathogen was controlled by the

bacterium applied to surface wounds of the fruit.
It is assumed that antibiosis are not involved in the mode of action (Droby et al., 1989; Chalutz and Wilson, 1990).

2. Competition for nutrients and space
In order to demonstrate that nutrient competition is a mechanism of antagonism, it is essential to show that the pathogenic fungi involved require an external source of nutrients in order to penetrate the living tissue.
We have reported (Droby et al., 1989) that the yeast antagonist Pichia guilliermondii (US-7) multiplied very rapidly at the wound site under a wide range of temperature, humidity and nutritional conditions and that it may increase in numbers, by 1 to 2 orders of magnitude, within 24 h. By the end of this incubation period the pathogen spores had just started to germinate and grow.
The growth rate of the yeast at the wound site at different temperatures and its persistency in the tissue is shown in Figure 1 A. The ability of the antagonist cells to rapidly increase in number at relatively low temperatures, compared with the pathogen, explains the observed increase in efficacy against the green mold of grapefruit when the efficacy is compared at decreasing storage temperatures (Fig. 1 B).

Figure 1 Growth and biocontrol activity of US-7 at different temperatures.





Several lines of evidence supported the assumption that the inhibition of the pathogen development by the antagonist involves competition for nutrients (Droby et al., 1989). Such competition was demonstrated by P. guilliermondii in culture, when both the antagonist and the pathogen were co-cultured in a minimal synthetic medium or in a wound leachate solution. On the fruit, the efficacy of the yeast could be markedly reduced by the addition of nutrients to the spore suspension used for inoculation.
Droby et al., (1991) have shown that the number of antagonist cells itself at the wound site will not always determine its efficacy. Our data suggested that active multiplication and growth of the US-7 yeast cells was required for the yeast to exhibit its biocontrol activity. This was demonstrated by using a mutant of P. guilliermondii which lost its biocontrol activity against Penicillium digitatum on grapefruit and Botrytis cinerea on apples, even when applied to the wound at concentrations as high as 10^{10} cells/ml. At the wound sites, the cell population of this mutant remained constant during the incubation period, while that of the wild type increased almost 60 to 100-fold, within 24 h. Failure of the mutant to inhibit spore germination of the pathogen in vitro on a minimal salt medium suggested that this mutant lost its ability to utilize nutrients and grow in culture as it did on the host. This could be the reason for its non-efficacy.

3. Induction of resistance mechanisms in the host

Several yeast antagonists of wound pathogens are most effective when their application occurs prior to, or simultaneously with inoculation by the pathogen. Application of the antagonist cells after inoculation resulted in decreased efficacy. The longer the time elapsing between infection and antagonist application, the less was the antagonist efficacy. While this trend has been demonstrated in all laboratory studies (Chalutz and Wilson, 1990), in larger scale semi-commercial tests, reduction of yeast efficacy due to its application after inoculation, although clearly evident, was much lower than exhibited in the laboratory tests (Table 1). These repeated observations suggested the possibility that application of the yeast cells may induce resistance processes in the peel tissue possibly at a distant site that could not be reached by the yeast cells.

Table 1 Effect of time of yeast application on biocontrol control activity.

Time of application after inoculation	Water control	US-7
(h)	(% Infected wound sites)	
0	43	10
3	60	18
6	77	23
24	86	44

To test this hypothesis, we examined the production of ethylene by yeast-treated tissue; when cell suspensions of the US-7 yeast antagonist were placed on surface wounds of grapefruit, pomelo, table grapes or carrot root tissue, stimulated ethylene production was evident in all tissues.

The involvement of ethylene in the induction of resistance processes in grapefruit and carrot roots was demonstrated in the past possibly through the induction of the activity of phenyalanine ammonia-lyase (PAL), an enzyme which catalyses the branch point step reaction of the shikimic acid pathway, leading to the synthesis of phenols, phytoalexins and lignins, all associated with induced resistance processes (Halbrock and Grisebach, 1979; Kuc, 1982). In citrus fruit, ethylene production and PAL activity were induced following application to peel discs of an effective yeast antagonis (Droby, et al., 1991), while exogenously applied ethylene to the discs or to the whole fruit, induced resistance to P. digitatum infections. Thus, the induction of ethylene production during the interaction of the antagonist with the tissue suggests the involvement of host resistance mechanisms in the yeast action. The nature of this mechanism is yet to be elucidated.

4. Direct interactions with the pathogen

Direct parasitism of the antagonist to the pathogens' propagules has been reported to play a role in biological control against soil borne and foliar diseases (Chet et al., 1979). In the postharvest arena, however, very little information is available on biological control agents that directly parasitise pathogens. Wisniewski et al. (1991) have shown that the yeast antagonist P. guilliermondii, when co-cultured with B. cinerea, appears to strongly attach to B. cinerea hyphae. This attachment was blocked when the yeast cells or the fungal hyphae were exposed to compounds that affect protein integrity, or when respiration was inhibited. A lectin-type binding was suggested in this attachment. In addition, P. guilliermondii was found to exhibit a high levels of

β-(1-3) glucanase activity when cultured on various carbon substrates or on cell walls of several fungal pathogens. This enzyme has been purified by us and its specific role in the mode of action of the yeast is now being evaluated on the molecular level. The fastidious attachment of P. guilliermondii to fungal cell wall would enhance the effectiveness of any cell wall hydrolases secreted by the yeast to the extracellular matrix. When yeast cells were dislodged from the hyphae, concave appearance of hyphal surface and partial degradation of B. cinerea cell wall was also observed at the attachment sites. Thus, the firm attachment of the yeast cell along with the production of hydrolases may be responsible for the observed degradation of fungal cell wall. Production of glucanase may also enhance the ability of the yeast to adhere to fungal hyphae, as suggested for Candida albicans (Notario, 1982)
It appears that the mode of action comprises a complex mechanism which could involve one or several of the following processes: antibiotic production, nutrient competition, site exclusion, induced host resistance and direct interaction between the antagonist and the pathogen.

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BIOLOGICAL CONTROL OF *PENICILLIUM HIRSUTUM* BY ANTAGONISTIC SOIL BACTERIA.

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Summary

From a randomly selected population of soil micro-organisms, nine bacterial strains with antagonistic activity towards *Penicillium hirsutum*, the causal agent of storage rot of tulip bulbs, have been isolated and characterized. Three have been classified as *Pseudomonas fluorescens* A, four as *Bacillus polymyxa* and two as *Bacillus* sp. The *B. polymyxa* and *Bacillus* sp. strains secreted active antifungal compounds in the growth medium. Compared to the *B. polymyxa* strains, the antifungal compound(s) produced by one of the *Bacillus* sp. strains, designated as G22, showed a distinct different specificity towards fungal pathogens. The antifungal activity of the various strains most likely originates from low-molecular weight compounds which were found to be highly thermostable.

Introduction

Storage rot of tulip and hyacinth bulbs, caused by the fungus *Penicillium hirsutum* [3], is regarded to be an important post-harvest disease. Generally, the chemical fungicide benomyl is applied for the control of *Penicillium* infection. However, several benomyl resistant *Penicillium* isolates from different sources have been detected [1]. These findings, and public and governmental pressure to reduce the use of pesticides, emphasize the necessity to develop alternative means of controlling post-harvest rot diseases of flower bulbs.

Huang et al. [2] have recently demonstrated that *P. digitatum* can be suppressed by the antagonist *Bacillus pumilis*. Wilson and Chalutz [4] have demonstrated the presence of several potential antagonistic micro-organisms towards *P. digitatum* and *P. italicum* in the habitat of these phytopathogenic fungi.

The aim of this study is to apply antagonistic micro-organisms or their (purified) fungicidal products as bio-control agents on tulip bulbs.

Materials and methods

Fungal cultures were obtained from "Centraal Bureau voor Schimmelcultures" (CBS), Baarn, The Netherlands. The fungi were cultivated on PCA (Oxoid, Unipath Ltd., Hampshire, England). The soil bacteria were selected from PCA plates and the fungi and yeasts from oxytetracycline-glucose yeast extract agar (Oxoid).

The selected soil bacteria have been identified using the Biolog Identification system (Biolog Inc., Hayward, USA) for Gram-positive and Gram-negative bacteria.

HPLC analysis of the antifungal compounds has been done on culture supernatant of bacteria grown on a chemically defined medium containing 0.5% (w/v) glucose, 20 mM potassium phosphate pH 7.0, 8.5 mM NaCl, 7.5 mM $(NH_4)_2SO_4$, 2.5 mM $MgSO_4$ and Vishniac trace elements.

Results and discussion

1. Isolation and characterization of antagonists.

From soil samples of fields which are reported to be suppressive towards several fungal diseases (including *Penicillium* infection), we isolated randomly a population of 297 bacterial strains, 22 fungi and 15 yeasts. These micro-organisms were subsequently assayed for their ability to inhibit *in vitro* growth of *Penicillium hirsutum* (data not shown). In this population, two strong- and seven moderate antagonistic bacterial strains were present. Furthermore, two strong antagonistic fungi could be recognized. Interestingly, a significant number of fungal strains was found to be sensitive towards *P. hirsutum*, suggesting that the latter fungus also produces antifungal compounds. Finally, none of the isolated yeasts showed antagonistic activity towards *P. hirsutum*. The two strong and seven moderate antagonistic bacterial strains were used for further study.

After Gram-staining, the bacterial strains were identified using the BIOLOG identification system. Three Gram-negative strains were classified as a *Pseudomonas fluorescens* subgroup A (Table 1). Four Gram-positive strains were identified as *Bacillus polymyxa*. The remaining two strains were found to belong to the *Bacillus* genus, but could not be identified at the species level.

TABLE 1. Identification of antagonistic bacteria isolated from three different sources. Selection of all strains was based on antagonism towards the pathogenic fungus *Penicillium hirsutum*.

Strain	Gram staining	Species	Source
G10	negative	*Pseudomonas fluorescens* A	Grass land
G53	negative	*Pseudomonas fluorescens* A	Grass land
G76	negative	*Pseudomonas fluorescens* A	Grass land
N1C	positive	*Bacillus polymyxa*	Narcissus[a]
N117	positive	*Bacillus polymyxa*	Narcissus[a]
N123	positive	*Bacillus polymyxa*	Narcissus[a]
T129	positive	*Bacillus polymyxa*	Tulip[b]
T229	positive	*Bacillus* sp.[c]	Tulip[b]
G22	positive	*Bacillus* sp.[c]	Grass land

[a] Bacterial strains were isolated from soil in which *Narcissus* bulbs had been grown the previous year.
[b] Bacterial strains were isolated from soil in which *Tulip* bulbs had been grown the previous year.
[c] These strains could not be identified to the species level with the BIOLOG system.

2. Secretion of antifungal compounds.

To investigate the antifungal activity of the selected bacterial strains, aerobically cultured cells were separated from the growth medium by centrifugation. Subsequently, the collected culture supernatant was sterilized and added to a solid medium on which growth of *P. hirsutum* was monitored by measuring the hyphal extension. Growth of *P. hirsutum* (ex Tulipa) on a solid medium is severely inhibited in the presence of an 8-fold diluted culture supernatant of *B. polymyxa* N1C

(data not shown). However, no growth inhibition was observed when the fungus was cultivated on medium supplemented with an equal amount of culture supernatant of a non-antagonistic isolate G8. Similar observations were made for the culture supernatants of *B. polymyxa* T129 and *Bacillus* strain T229.

The culture supernatant of *Bacillus* sp. G22 hardly affected growth of *P. hirsutum* but instead severely inhibited growth of *Fusarium oxysporum* f.sp. *narcissi* (Fig. 1C).

These results show that the antifungal activity of the tested strains is associated with the culture supernatant indicating that an active, fungicidal compound is secreted in the growth medium. In addition, *Bacillus* sp. G22 displays a distinct specificity towards target fungi.

3. Characterization of antifungal activity.

The antifungal activity of strains N1C, T129 and G22 was measured at different pH-values. Culture supernatant of *B. polymyxa* N1C was found to be active against *P. hirsutum* ex Tulipa over a broad pH-range (Fig. 1A). In addition, supernatant of *B. polymyxa* T129 displayed the highest antifungal activity at high pH-values, with a peak activity at pH 9 (Fig 1B). Finally, supernatant of *Bacillus* sp. G22 exhibited full activity against *F. oxysporum* between pH 3 and 10 (Fig. 1C).

The thermostability of the antifungal compounds was studied by boiling the supernatant of strains T129 (*B. polymyxa*), N1C (*Bacillus polymyxa*), T229 (*Bacillus* sp.) and G22 (*Bacillus* sp.) for 60 minutes. In all cases the supernatant retained full activity (data not shown), indicating that the secreted antifungal compounds are highly thermostable and are presumably secondary metabolites.

Finally, ultrafiltration experiments indicated that the molecular weight of the antifungal compound(s) from strain T129 is (are) below 1 kD.

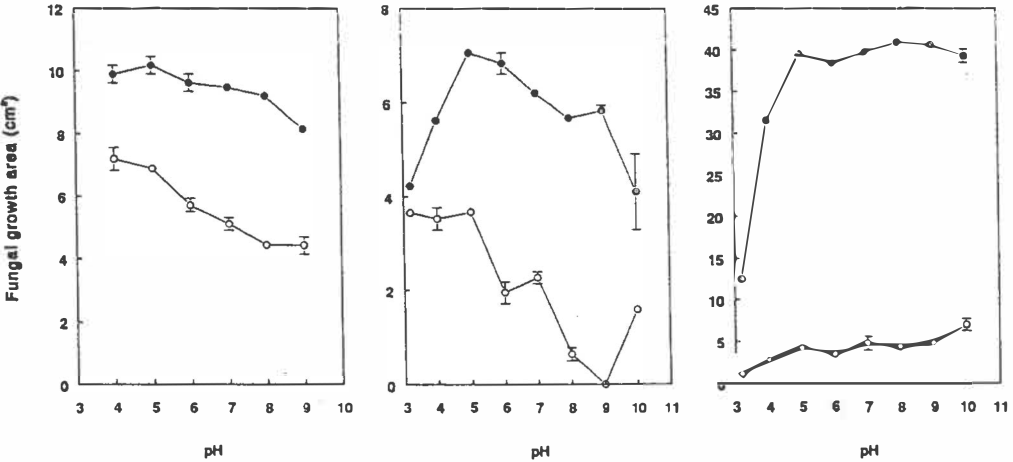

Figure 2. pH dependence of antifungal activity of culture supernatants of *B. polymyxa* N1C (panel A), *B. polymyxa* T129 (panel B) and *Bacillus* strain G22 (panel C). The activity of both *B. polymyxa* strains was assayed on *P. hirsutum* CBS 349.75 (ex Tulipa). The activity of *Bacillus* strain G22 was assayed on *F. oxysporum* f. sp. *narcissi* CBS 196.65. The fungi were grown on a chemically defined solid medium containing a citrate/MES/HEPES polybuffer. The medium was supplemented with 12.5 % bacterial culture supernatant (O). As a control, the growth medium was supplemented with 12.5% bacterial growth medium (●)

4. Isolation of antifungal compounds.

To isolate the antifungal compounds produced by the antagonistic bacteria, culture supernatants were concentrated by vacuum evaporation. Next, the concentrated samples were separated on a reversed-phase HPLC column and analyzed by monitoring the absorbance at 254 nm. It was found that *B. polymyxa* T129 secreted several different UV absorbing compounds into the growth medium (Fig. 3). The position of peaks obtained with culture supernatants from *B. polymyxa* N1C and *Bacillus* sp. T229 was qualitatively comparable. *Bacillus* sp. G22 supernatant, on the other hand, gave a completely different pattern of UV absorbing peaks (data not shown). This observation is in agreement with the notable difference in specificity of the antifungal activity.

Currently, we are developing a bio-assay, which can be used for the isolation, purification and chemical characterization of the active compounds secreted by the various antagonistic bacteria.

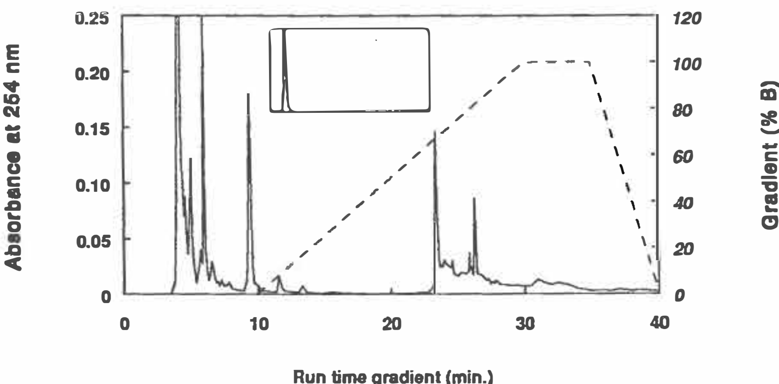

Figure 2. HPLC analysis of *B. polymyxa* T129 supernatant. Inset (same scale) represents a control run with the medium alone. The column was eluted with phosphate buffers containing 1% (buffer A) and 60 % acetonitril (buffer B).

Conclusions

At least four of the isolated *Bacillus* strains produce one or more thermostable, presumably low molecular weight antifungal compounds. For future application it is important to notice that the antifungal compounds produced by *B. polymyxa* N1C and *Bacillus* sp. G22 are highly active over a broad pH-range. The application of these antagonistic bacteria and their antifungal metabolites to control storage rot of tulip bulbs is currently being investigated.

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EFFECTS OF FATTY ACIDS ON THE IN VITRO RELEASE OF CUTINASE ACTIVITY AND ON THE INFECTION OF PLANT PATHOGENIC FUNGI

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Abstract
Esterase and cutinase activities were studied in *Mycosphaerella pinodes* (Berk. et Blox.) Verstergr., the agent of pea leaf spot, and in *Ascochyta pisi* Lib. *In vitro*, cutinase activity was induced by cutin, cutin hydrolysate, ethered extract of cutin hydrolysate (ECH). Cutin culture medium supplemented with ECH (0.3%), or juniperic acid (0.5%) as sole source of carbon inhibited the release of esterase and cutinase activities in the culture filtrate, whereas 0.5% ricinoleic acid added to the cutin culture medium, inhibited the induction of cutinase. A similar esterase-inhibiting effect was observed *in vitro* upon treatment of *Botrytis cinerea* with ricinoleic acid. *In planta*, non fungitoxic concentrations of ECH, added to the inoculum, protected pea leaflets against infection by *M. pinodes*. Infection was restored by wounding, or by adding exogenous cutinase to the inoculum, thus suggesting that the fatty acid-induced inhibition was due to interference with cutinase induction and/or release.

Introduction

Biocontrol preparations with agricultural potential should possess several desirable characteristics such as ease of preparation and application, good stability, and viability of the propagules. Formulations could also contribute to enhance the antagonistic activity of biocontrol agents (BCA) by coupling it to the protective effects of non-toxic molecules interfering with host-parasite interactions. Among the various inhibitory mechanisms involved, is the prevention of host penetration by disturbing cutinase activity.
In this respect, the first step of the infection process of many pathogenic fungi is the penetration through the cuticle. This cuticle is rich in cutin, a structural component which is an insoluble polymer made of hydroxy and hydroxyepoxy-fatty acids of the C-16 and C-18 families.
A model, describing the involvement of cutinase in the penetration process of fungi in host plants, has been developed for several plant pathogens, as summarized by KOLLER (1991). Inhibitory disturbances of cutinase induction and/or activity may prevent penetration in (and infection of) the hosts by several pathogenic fungi.
In the present work, we studied the effects of fatty acids on the *in vitro* release of cutinase and on the infection by

Mycosphaerella pinodes (Berk. et Blox.) Vestergr. [*Ascochyta pinodes*] and *Ascochyta pisi* Lib., two pathogens of pea leaves.

Results and Discussion

Upon *in vitro* culture, using glucose as sole carbon source, no cutinase was released by these fungi into the culture medium, as measured by fatty acids microtitration or evaluated by esterase activity (NASRAOUI, 1992).
When cutin (0.2%) was added to the modified Czapec-Dox mineral medium as sole carbon source (DICKMAN & PATIL, 1986), cutinase (as revealed by esterase activity) was induced and released by germinating spores and growing mycelium after 72h of incubation (table 1). Post treatment of such culture with acetone (20% v/v, during 2h), used as an enzyme extractor, enhanced the release of the esterase activity in the medium.
When 0.3% of ethered extract of cutin hydrolysate (ECH), prepared according to the method of LIN & KOLATTUKUDY (1978), was added to the culture medium containing 2g/l of cutin, the released esterase activity lowered, but was restored upon post treatment with acetone (table 1). The esterase activity extracted after acetone post treatment suggests that, in the presence of inhibitory concentrations of ECH, the enzyme is induced, but not released in the culture medium.
Similar results were obtained using the commercially available juniperic acid (16-hydroxyhexadecanoic acid) as cutinase inducer. Upon incubation of *A. pisi* for 72 h on mineral culture medium supplemented with increasing concentrations of juniperic acid as sole carbon source, the release of esterase activity reached a peak at 0.05%. Release was reduced but induction remained unchanged at higher fatty acid concentration (0.5%). When juniperic acid (0.05% or 0.5%) was incorporated to the culture medium together with cutin, release remained low too (but induction was present).
When culture medium containing cutin was supplemented with high concentrations of ricinoleic acid (or [R]-12-hydroxy-cis-9-octadecenoic acid, obtained by extraction of castor bean), esterase activity released by *A. pisi* after 24h remained low, whatever the post treatment (acetone or water) (figure 1). Similar results were obtained with *Botrytis cinerea* (figure 2), a pathogen for which cutinase was also shown to be implied in the penetration of gerbera flowers, and whose infection was stopped with specific anticutinase antibodies (SALINAS, 1992).
The effect of ECH on the infection of pea leaflets by *M. pinodes* was investigated, using the biological assay previously described (NASRAOUI, 1992). Supplementing *M. pinodes* inoculum with ECH (at 0.1%, a non fungitoxic concentration) inhibited the infection of unwounded pea leaflets, but not that of Carborundum-wounded leaflets. Addition of a dialysed filtrate of *M. pinodes* grown on cutin culture medium (and containing cutinase activity), to the *M. pinodes* inoculum supplemented with inhibitory concentrations of ECH, restored infection of unwounded leaflets (table 2). In contrast, addition of a dialysed filtrate of *M. pinodes* grown on glucose culture medium

Carbon source in the culture media	ESTERASE ACTIVITY OF CULTURE FILTRATE			
	spontanous release		acetone post treatment	
	A.pisi	*A.pinodes*	*A.pisi*	*A.pinodes*
Cutin 0.2%	2.4	1.2	4.7	3.9
Cutin 0.2% + 0.3% ECH°	0.8	0.4	4.6	3.8
Juniperic acid 0.05%	2.1	-	4.9	-
0.5%	1.0	-	4.8	-

°ECH= Ethered extract of cutin hydrolysate (fatty acids from apple cutin).

Table 1: Esterase activity (U/ml) of the culture media (72h of incubation).

(containing almost no cutinase activity), did not restore infection. This result suggests that cutinase action was inhibited by high concentrations of cutin fatty acids, and so penetration in -and infection of- unwounded pea leaflets by *M. pinodes*.
The protection effect of ECH (concentration up to 0.3%) on infection of unwounded pea leaflets was not observed with *A. pisi*, probably because of the high inoculum concentration needed to provide infection and due to the constitutive cutinase thus released by the germinating spores of this fungus.
It is already known that fatty acids may protect plants against infection, either through the induction of resistance mechanisms in the plant (COHEN *et al.*, 1991), or by direct fungitoxicity.
Our overall results suggest that some fatty acids possess specific properties related to the induction and release of cutinase by pathogenic fungi and could be used to enhance the protective effects of BCA through these antipenetrant activities.

This work was financially supported by the French Community of Belgium, Contract ARC 90/94-143.

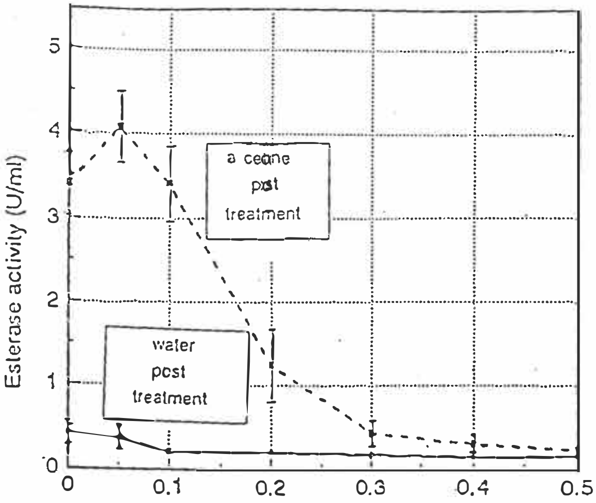

Figure 1: Influence of ricinoleic acid on *Ascochyta pisi* esterase activity induced in cutin medium, after 24h of culture.

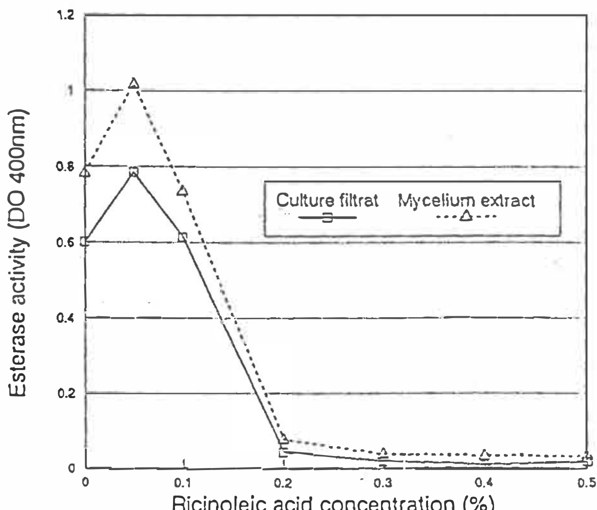

Figure 2: Influence of ricinoleic acid on *Botrytis cinerea* esterase activity induced in cutin medium, after 72h of culture.

Treatment	Symptoms intensity of pea leaflets after 48h (0-3 scale) (NASRAOUI *etal*.1990)
Inoculum	3
Inoculum + 0.1% ECH°	0
Inoculum + 0.1% ECH (wounded leaflets)	3
Inoculum + 0.1% ECH + Exogenous cutinase°°	2.4

°ECH= Ethered extract of cutin hydrolysat (fatty acids from apple cutin)
°° Cutinase of *M.pinodes* grown in a cutin (0.2%) culture medium

Table 2: Effects of fatty acids on pea leaflets infection by *M.pinodes*

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BIOLOGICAL CONTROL OF BOTRYTIS CINEREA BY PREMUNITION.

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Summary
A new strategy for biocontrol of Botrytis cinerea is proposed here. The fungus would be stopped by the plant itself, because of the premunition of the plant. Our proposal only concerns plant tissues 'with resistance'. We hypothesize that a low-pathogenic fungus in such tissues would trigger the defence mechanism with a minimal of necrosis. The raised defence would constitute a preformed barrier for the subsequently invading wild-type strains. We report here arguments to create the low-pathogenic strain by elimination of the polygalacturonase activity.

Introduction

Lesions caused by Botrytis cinerea keep restricted or get expanding in the plant tissues. The size of the lesions especially depends on the weather conditions, the type of plant tissue and the start inoculum.
We hypothesize that a B. cinerea strain low in pathogenicity could be used for premunition of a number of plant tissues. Such a B. cinerea strain should be inoculated on wounds or on senescent or necrotic tissues. It would bring about restricted lesions since the plant defence system would be elicited. Subsequent wild type B. cinerea infections would be unable to cause expanding lesions.
For this hypothesis final proof can be given only in case a low-pathogenic B. cinerea strain is available. Here we report the results of a number of our experiments giving lines of evidence for the plausibility of the hypothesis.

Results

1. Observations at the penetration in plant tissues.
Most infections arise from conidia. We distincted two extreme situations :
(a) The intact epidermis is penetrated following the germination. Soon the killing of the underlying tissue starts. This was observed e.g. in different petals.
(b) The infections are inhibited in various ways : e.g. there is no germination, or no penetration, or after germination and penetration the lesions are restricted. Such host tissues have some capacity to suppress the penetration of the pathogen. Successful infections are possible only if the conidia can germinate on wounds or on a 'saprophytic starting base'.

2. Observations of mycelium growth in lesion expansion.
At the margins of the lesions - according to the type of plant tissue - two types of B. cinerea expansion were observed.
(a) In some tissues a front of hyphal tips is growing among the healthy cells without hindrance, and the host cells are killed hereafter. This was seen e.g. in strawberry petals.
(b) In other tissues such as green leaves and immature fruits the fungus, which penetrated via a wound or some other preformed lesion, is apparently unable to grow between the healthy cells. The cells of the invaded tissue are killed ahead of the hyphal tips and the fungus is following the progressing necrosis. In this case the host cells try to resist the fungal growth. We defined such tissues as 'tissues with resistance'.

3. Symptoms at the border of restricted or expanding lesions in tissues with resistance
Regarding the fungal invasion of 'tissues with resistance' three major zones are marked out (table 1).

Table 1. Observed zones at the border of the lesions.

zones :	0	Ia / Ib	II	III
	HYPHAL TIPS	NECROSIS	DEFENCE	HEALTHY
1. expanding lesions	growing between cells killed ahead	no / still plasmolysis intracell.desorgan.	weak * fluoresc.	no any effect visible
2. restricted lesions	idem	dark brown necrosis	bright * fluoresc.	idem

* in plants with fluorescent phytoalexins

The elicitation of defence occurs in the living cells at the border of the lesion (Mansfield, 1980). A whole battery of defence compounds are involved in this process, among which phytoalexins. Some plants possess fluorescent phytoalexins. We correlated the weak or bright fluorescence in the so-called zone II with the defence reaction of the plant. The brown necrotic tissue ahead of the hyphae in expanding lesions is characterized by the inability of the cells to plasmolyse. However, at the most remote border (Ib) the contents of the cells are desorganized at first while still capable of plasmolysis. We showed that each of both necrotic symptoms is caused by another secretion.

4. Role of different secretions of B. cinerea in necrosis and defence.
4.1 Factors of necrosis.
(a) Kamoen et al. (1978) examined the course of symptoms in leaves infiltrated with and incubated in pectic enzyme preparations of B. cinerea. One of the first symptoms seen (after three hours of incubation in pectic enzyme) was the inability of the cells to plasmolyse in hypertonic sucrose solution, a criterium for cell death. Only later the cellular organels were desorganised.
(b) Two groupes of polysaccharides are secreted by B. cinerea viz the glucans (M.W. > 10^6) and the lower-weight rhamno-galacto-mannans (M.W. < 50.10^3). When leaves were incubated in B. cinerea rhamno-galacto-mannans, necrosis occured too, but not during the first hours. The cells retained their capacity to plasmolyse until some eight hours after incubation. One of the first symptoms here was the desorganisation of cellular organels (after two hours), as was observed by the changes in shape and size of the chloroplasts. At high concentration and constant supply the phytotoxic activity of the polysaccharides is clearly shown.

4.2 Factors of defence.
(a) In our opinion the pectic enzymes of B. cinerea cannot or can only in part account for the elicitation of defence substances. At our lab expanding lesions were created in moist conditions on grape leaves which then were infiltrated either with pectic enzyme or with water and kept moist for a further 16 hours. When the leaves were then transferred to dry circumstances, a clear border of fluorescent phytoalexins around the lesions was monitored only in the water-infiltrated leaves. In leaves treated with polygalacturonase, fluorescence was less and was weakly scattering over the whole surface of the leaves.
(b) Kamoen et al. (1980) separated and purified glucans and mannans of B. cinerea in order to test for their capacity as elicitors for the defence. Bean leaves were infiltrated under vacuum with either type of polysaccharide and incubated in humidity. The amount of phaseollin was measured during several days at time intervals of 24 hours. It was concluded that the rhamno-galacto-mannans caused increased levels of phaseollin compared to the control (water). The concentrations applied of those polysaccharides and the concentrations rated of phytoalexins were closely correlated. It is concluded that the mannans besides their killing effect (4.1,b) represent the first-line elicitors of defence, especially when present in sub-toxic concentrations. Kamoen and Dubourdieu (1990) studied polysaccharide contents in diseased grapes compared to healthy grapes. The in vivo presence of B. cinerea glucans and

mannans was confirmed. Thus the hypothesis that they are secreted during the infection process is supported.

5. Reasons for expansion and restriction.
The zones reported in table 1 can be correlated with the differential actions of the B. cinerea secretions. Zone I would be the result of the phytotoxic actions of pectic enzymes and polysaccharides. The effect of polysaccharides (zone Ib) is reaching further than the effect of polygalacturonase. Up to now there are only hypotheses to explain it. Zone II would arise in response to subtoxic levels of the elicitors (mannans). The elicitation of defence as well as killing of the cells probably depends on the diffusion of substances into the undamaged tissue (Kamoen, 1989). The water content of the intercellular spaces influences much the spread of the lesions. At high humidity zone I is expanding very fast. Polygalacturonases together with higher concentrations of polysaccharides are killing the tissue. Then in zone II the phytoalexins (and other defence compounds) would not be able to accumulate up to the level of effectiveness. Under dry circumstances most lesions get restricted, possibly due to the limited diffusion. At the margins of the lesions, probably due to the subtoxic level of mannans, defence is elicited. Substances are synthesized de novo in the living border cells and would be able to attain higher levels and create a real defence barrier. This would explain the bright fluorescence (of phytoalexins) observed around restricted lesions.

Discussion

Based on our observations and results and based on the results reported in literature, we formulated a proposal to control B. cinerea. It is founded on the capacity of some plant tissues to resist B. cinerea invasion. For our biocontrol system a low-pathogenic strain is the first requisite. Although several secretions are mentioned to be responsible for the pathogenesis, we propose to create this strain by elimination of the polygalacturonase (PG) activity. Evidence exists for the phytotoxicity (inability of cells to plasmolyse) and for the presence in plant-pathogen interactions. On the other hand, according to the mode of action of pectic enzymes the synthesis of elicited defence substances is interrupted. The mannans however are acting as elicitors in the first place, and become phytotoxic only at higher concentrations or after a longer period of action. Therefore we are expecting that a PG⁻ strain would colonise wounds and 'saprophytic starting bases', and would be unable to kill the living tissues. The mannans that still would be secreted, would elicit the defence mechanism. In this way the plant tissues possibly obtain 'premunition' against aggressive wild-type strains.

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INDUCED RESISTANCE IN BARLEY TOWARDS POWDERY MILDEW AND THE INFLUENCE ON SINK-SOURCE RELATIONSHIPS

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Summary

The induction of resistance in barley plants with microbial metabolites produced by a *Bacillus subtilis* strain leads to a reduced infection rate with *Erysiphe graminis* but, infection is never prevented completely. In field experiments the remaining mildew on induced resistant plants did not affect yield formation. The reduced damaging effect of the powdery mildew also became obvious in high carbohydrate contents of the grains and the translocation rate of ^{14}C from the flag leaf into the ear which was comparable to that in non-infected plants. Induced resistant barley plants infected with the same number of colonies showed a greater accumulation of sucrose in the leaf tissue under greenhouse conditions. In non-induced plants sucrose concentrations were highest underneath mildew pustules whereas on induced leaves the colonies were not able to accumulate comparable amounts of sugar in the direct vicinity. In addition conidia formed on induced resistant barley contained less carbohydrates. Obviously the powdery mildew was not able to build up an effective sink on induced resistant plants.

Introduction

The induction of resistance leads to a decreased susceptibility of plants towards diseases without changing the genetic constitution of the plant. Instead of eliminating the pathogens plants are stimulated to defend themselves by metabolic changes. Resistance to obligate biotrophic fungi can be induced by the application of microbial metabolites produced by a *Bacillus subtilis* strain (SCHÖNBECK et al., 1980).
On induced resistant barley the number and size of mildew colonies, the reproduction rate and the efficiency of haustoria are reduced (Stenzel et al., 1985) but, infection is never prevented completely.
In field experiments the remaining mildew on induced resistant barley plants did not affect yield (Steiner et al., 1988). This paper presents results of experiments conducted in the field and in the greenhouse to find out whether the carbohydrate metabolism is influenced by the induction of resistance and in addition if sink-source relationships are changed in resistant plants.

Materials and Methods

The winter barley cultivar 'Mammut' was used as host plant for *Erysiphe graminis* DC f.sp. *hordei* (Em. Marchal). Resistance was induced with a purified protein rich fraction isolated from the culture filtrate of a *Bacillus subtilis* strain (B 50, Schönbeck et al., 1980).

The experiment in the field was carried out in 1991 in Hannover. Each treatment was replicated four times randomized on the experimental area. Plants were naturally infected with powdery mildew while non-infected control plants were treated with the fungicide 'Desmel' (500 ml/ha). The inducer was applied three times (EC 59, 65 and 75) according to the development of disease. Powdery mildew infection was estimated on the upper three leaves of 30 main tillers (Oerke et al., 1989). Carbohydrate contents of grains were measured photometrically (Oerke, 1988). Barley plants for ^{14}C-translocation experiments were cultivated in mitscherlich pots in a compost substrate with 10 plants per pot. Radiolabelling was carried out in stage of early milk in the lab (Böhnke, 1992).

In the greenhouse barley plants were grown in a sterilized compost at day/night temperatures of 21/17°C and a photo period of 16 h. Secondary leaves were inoculated (EC 12) by spraying mildew conidia onto the leaf. Non-infected control plants were treated with 30 ml Ethirimol ('Milgo-E', 0,2 %). Seven dpi mildew pustules were counted and for gaschromatographical estimation of soluble sugars (Green, 1983) only plants infected with the same number of colonies were selected. The inducer was sprayed onto the plants two days before inoculation. Control plants were treated with an aliquot of sterilized non-active inducer solution. Soluble sugar contents of conidia were also analysed by gaschromatography, total carbohydrate amounts according to Umbreit et al. (1972).

Results

The infection density of barley with powdery mildew was reduced by the inducer treatment (Tab. 1). Although the pathogen still remained on the plant the yield of induced resistant plants was not affected and exeeded that of fungicide protected plants.

TABLE 1: Effect of induced resistance in barley towards powdery mildew on yield, infection density and carbohydrate contents of grains

treatment	yield [dt/ha]	rel.	% mildew EC 82	[mg starch/ grain]
untreated	85,0	100 %	66	18,44
Inducer	102,0	120 %	39	25,54
Fungicide	97,8	115 %	7	23,79

The observed reduction of the damaging effect of powdery mildew on induced resistant plants also became obvious in the carbohydrate contents of the grains which were the same level as those of fungicide treated plants.
Flag leaves of untreated barley plants accumulated a greater percentage of radiolabelled carbohydrates (Fig. 1). In contrast to that treatments with the inducer and the fungicide led to a higher amount of ^{14}C-assimilates translocated into the ear. On induced

resistant plants infected with mildew the supply of the ear with carbohydrates was as high as on non-infected barley plants.
For comparing the sink of powdery mildew on induced and untreated barley plants individuals grown in the greenhouse infected with the same number of mildew colonies were examined.

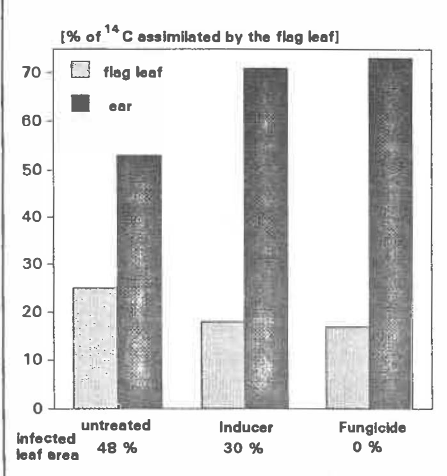

Fig.1: Effect of induced resistance on the translocation of radiolabelled metabolites into the ear of mildew infected barley

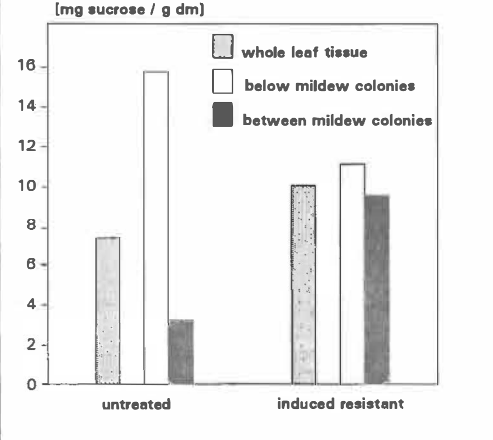

Fig.2: Effect of induced resistance on sucrose contents of barley leaves below and between mildew colonies

On induced resistant plants mildew infection resulted in a higher increase of sucrose in the whole leaf (Fig.2). For localizing the sucrose within the leaf mildew colonies were punched out and the tissue between and underneath pustules was analysed. In contrast to untreated ones the induced resistant plants showed a decreased accumulation of sucrose below colonies. Instead a larger amount of sucrose was found in the uninfected regions of the leaf.

TABLE 2: Carbohydrate contents of powdery mildew conidia formed on induced resitant barley

	untreated control [µg/conidia]	induced resistant [µg/conidia]	rel to untr.
dm/conidia	$3{,}74 \times 10^{-2}$	$3{,}31 \times 10^{-2}$	88,5 %
total carbohydr.	$1{,}89 \times 10^{-3}$	$1{,}30 \times 10^{-3}$	68,8 %
fructose	$1{,}68 \times 10^{-4}$	$1{,}29 \times 10^{-4}$	76,8 %
arabitol	$1{,}37 \times 10^{-3}$	$1{,}18 \times 10^{-3}$	86,1 %
trehalose	$4{,}55 \times 10^{-4}$	$4{,}12 \times 10^{-4}$	90,5 %
mannose	$4{,}24 \times 10^{-5}$	$3{,}80 \times 10^{-5}$	89,6 %

In addition mildew conidia formed on induced resistant barley plants contained less carbohydrates than those formed on untreated control plants (Tab. 2). The dry matter per conidia was reduced as well as total carbohydrates, fructose and fungal specific sugars like arabitol, trehalose and mannose.

Discussion

The results presented indicate that conditions for the development of powdery mildew on induced resistant plants are very poor. The fungus accumulates sucrose in the leaf tissue but obviously on induced resistant plants the mildew is not able to take up the same amount of sugar as on non-induced barley plants. The sucrose remains in the leaf and probably can be used by the plant for building up plant tissue. This results in increased yield. Presumably the ear as a sink-organ can compete much better with the remaining mildew for the assimilates and therefore grains are supplied much better.
These alterations in sink-source relationships could be an explanation for the higher tolerance of induced barley plants towards remaining mildew infection.

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INDUCTION OF DISEASE RESISTANCE AGAINST BARLEY POWDERY MILDEW, BIOLOGICAL AND MOLECULAR BACKGROUD AND PRACTICAL IMPLICATIONS

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Introduction

The induction of resistance (IR) following inoculation with the same or other microorganisms is well known from many host-pathogen interactions. IR can be defined as active defence based on physical and chemical barriers which acts against subsequent infections by otherwise pathogenic organisms. IR is elicited by inoculations with pathogens, non-host pathogens and saprophytes, metabolic products from such organisms or different abiotic stimuli. This paper gives an account of recent results concerning the resistance induced locally in barley against barley powdery mildew fungus.

Results

Induction of resistance against barley powdery mildew

A series of experiments was conducted in order to test the resistance-inducing capacity of various microorganisms on barley leaves against challenge infection with a virulent race of the barley powdery mildew fungus (Erysiphe graminis f.sp. hordei, Egh). The results showed that both avirulent and virulent races of Egh as well as the non-host pathogen E. graminis f.sp. tritici (Egt), the saprophytic fungus Cladosporium macrocarpum and the bacterium Bacillus subtilis are strong inducers of resistance (Cho & Smedegaard-Petersen, 1986; Christiansen & Smedegaard, 1990; Smedegaard-Petersen *et al.*, 1991; Thordal-Christensen & Smedegaard-Petersen, 1988a,b).

The degree of IR depends on the inoculum density of the inducer and on the period of induction. Conidia of saprophytic fungi, the non-host pathogen Egt and the pathogen Egh germinate and form appressoria on barley leaf surface. Appressoria produce infection pegs which attempt to penetrate the epidermal cell wall. The attempted penetration elicits distinct reactions in the epidermal cells including the aggregation of the cell contents and development of papillae under the penetration sites (Gregersen & Smedegaard, 1989; Thordal-Christensen & Smedegaard-Petersen, 1988b). By using powdery mildew as the inducer, an induction period of less than 1 hour caused significant reduction in the number of mildew colonies produced by the challenger, and an induction period of 6 hours resulted in 70-75% reduction of mildew colonies. The very rapid host response may indicate that the primary germ tube is involved in induction since it establishes a physical relationship with the epidermal cells of the host within one hour of inoculation (Thordal-Christensen & Smedegaard-Petersen, 1988a,b). Using B. subtilis as an inducer, the bacterial cells and culture supernatant are capable of reducing mildew attack. The capacity of different bacterial culture fractions to IR was unaffected by heat treatment (Wei *et al.*, in prep.).

Physiological and biochemical changes associated with induced resistance in barley

The energetic requirement for IR has been investigated. With a single inoculation, the resistant barley leaves showed a rapid temporary respiratory increase within 8 hours after inoculation which returned to the level of the non-inoculated control after three days (Smedegaard-Petersen & Stølen, 1981). Repeated inoculation of barley plants with an avirulent race of Egh caused a permanent increase in the rate of respiration and a concomitant reduction in yield, even though the plants did not show any visible disease symptoms. Furthermore, in virtually disease-free field plots, fungicide treatments which suppressed the saprophytic flora on the leaf surfaces resulted in increased yield (Smedegaard-Petersen *et al.*, 1991). These results suggest that, in plants grown in nature, a general response is more or less constantly activated by the interaction of the plant with surface microbes.

Larger fluorescence papillae are induced by challenge inoculation of barley plants exhibiting IR than in controls (Thordal-Christensen & Smedegaard-Petersen, 1988b). Papillae and haloes thus seem to act as a physical or chemical barrier which reduce challenge infection. Histochemical staining of epidermal tissue from inoculated barley and wheat leaves demonstrated a high content of callose and polyphenolics in papillae, whereas there is only a weak accumulation of these compounds in the haloes surrounding the papillae. Phloroglucinol-HCl stain which indicates the presence of lignin gave a strong positive reaction with papillae and haloes in wheat but only a weak reaction with papillae and haloes in barley. By the use of Sakaguchi-reagent stain which indicates guanidine groups, we have detected, for the first time, substantial accumulation of guanidine-containing compounds in papillae and related cell walls of powdery mildew-infected barley leaves, whereas there was a weak reaction for such compounds in papillae of infected wheat leaves (Wei *et al.*, in prep.).

Molecular changes associated with induced resistance in barley

Two-dimensional polyacrylamide gel electrophoresis of in vitro translation products of mRNA isolated from barley leaves indicated an early and rapid activation of a specific set of genes in barley leaves inoculated with Egt compared to uninoculation leaves (Bryngelsson & Collinge, 1992; Gregersen *et al.*, 1990).

In order to clone these induced response genes, two cDNA libraries were constructed using mRNAs extracted from barley leaves 6 hours after inoculation with an avirulent race of Egh and 72 hours after inoculation with a virulent race of Egh, respectively. Differential or subtractive hybridization techniques allow the selection of clones which represent genes whose transcripts accumulated following inducer inoculation. Nine cDNA clones were selected and characterized by sequencing. The putative identity of several sequences was suggested by homology searches of data bases (Brandt *et al.*, 1992; Bryngelsson & Collinge, 1992; Thordal-Christensen *et al.*, 1992; Waither-Larsen *et al.*, 1992) (Table 1), and only one of these was obtained by Davidson *et al.*, 1987.

Discussion

Plant surfaces are often colonized by bacteria, fungi and other phyllosphere microorganisms. These microorganisms stimulate plants to react with energy-consuming defence reactions which, on the one hand, protect the plant against subsequent pathogen-attack, and, on the other hand, often result in yield reduction (Smedegaard-Petersen & Tolstrup, 1985). This dynamic process illustrates the complex interactions among host, pathogen and environment. In practical disease control, by spraying barley and wheat crops with culture filtrates from the bacterium B. subtilis, a significant reduction of attacks by powdery mildew and a significant

increase in grain yield were recorded (Stenzel *et al.*, 1985). Under laboratory conditions, the results from our laboratory showed that spraying with culture fractions of a selected strain (KA-5) of B. subtilis on barley first leaves resulted in 70% reduction in the number of colonies of barley powdery mildew. Our results indicate that the protecting capacity of B. subtilis is due partly to an antagonistic effect by antibiotic compounds produced by the bacterium, partly to new increased expression of host resistance genes elicited by the bacterium. Northern blot hybridization studies revealed that a transcript representing a peroxidase sequence encoded by pBH6-301 accumulated in barley leaves pretreated with B. subtilis culture suspension, culture supernatant and bacterial cells (Wei *et al.*, in prep.).

TABLE 1. Isolated cDNA clones which represent gene transcripts from barley induced following inoculation with Erysiphe graminis f.sp. hordei. + - ++++: relative accumulation of gene transcripts observed in northern blot hybridisations to RNA from different host-pathogen interactions.

cDNA clones	related clones (nos.)	sequence homology	induction by E. graminis: early interactions	late compatib. interaction
pBH6-301	1	peroxidase [Thordal-Christensen *et al.*, 1992]	++++	++++
pBT6-4	1	sucrose synthetase	+	+
pHV1433a	3	14-3-3 protein from mammals. Putative regulatory protein of signal transduction [Brandt *et al.*, 1992]	++	-
pBH6-601	1	GRP94, ER-resident member of the HSP90 family [Walther-Larsen *et al.*, 1992].	+++	+++
pBH6-12	2	none	++++	++++
pBH72-A6	2	PR-1 protein (tobacco)	++++	++++
pBH72-B8	2	PR-4 protein (tobacco)	++++	++++
pBH72-C6	4	Hv-1, thaumatin-like PR-5 protein	++++	++++
pBH72-F1	1	weak similarity to *O*-methyl-transferases	+	++++

Papilla and halo formation activated by inducers seems to be the major physical or chemical barriers preventing the challenge infection. The drop-diffusate tests for phytoalexin extraction was not successful in isolating antimicrobial compounds from infected barley or wheat leaves. Consistent with this result, methanol extracts from barley leaves inoculated with Egh did not show any increased antimicrobial activity when compared to those obtained from uninoculated leaves (unpublished results). Thus, free antimicrobial compounds (phytoalexin-like compounds) apparently do not accumulate in induced papilla and halo structures. Histological studies indicate that the guanidine derivatives accumulate in papillae and adjacent cell walls. Compounds containing guanidine groups such as hordatines (the preformed alkaloids in barley) and streptomycin are highly antimicrobial. It is therefore suggested that the guanidine derivatives bound to papillae and related cell walls (interfering with insoluble lignin structure) may act as a resistance factor in induced resistance of barley.

From molecular studies, several genes have been isolated. Some of them show interesting functions which may be related to the regulation of active defence responses of barley plants (Brandt *et al.*, 1992; Thordal-Christensen *et al.*, 1992; Walther-Larsen *et al.*, 1992). More induced defence response genes are being isolated. The biochemical function, tissue localization, regulation of expression and role of these genes in resistance will be studied.

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INDUCTION OF RESISTANCE IN HOST PLANTS OF FOLIAR DISEASES VIA APPLICATON OF PLANT EXTRACTS

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Summary

Induced resistance can be achieved by application of plant extracts. One plant already investigated in detail is the giant knotweed, Reynoutria sachalinensis. An aqueous extract gave protection against powdery mildew fungi on various host plants. Other plant extracts were tested against Phytophthora infestans on tomato. In a first screening 37 out of 47 extracts kept infection rates below 15%. The presence of light was found to be a necessity to reach this kind of protection against the fungus. For one extract the lack of any curative effect could be shown whereas prophylactic treatment resulted in a protection of at least 7 days.

Introduction

Induced resistance is one of the strategies that can be used in biological control of foliar diseases. There are various reports on induction by culture filtrates, salts or other substances. In most of these cases the resistance is accompanied by hypersensitive responses (HR) and the accumulation of phytoalexins. This means, plants become weaker by partial leaf necrosis.
In opposite to that induced resistance can be achieved by plant extracts without leading to HR. A plant which has been investigated in detail with respect to resistance induction against powdery mildew fungi is the giant knotweed, Reynoutria sachalinensis. Other extracts have been tested against Phythophthora infestans.

1. Induced resistance against powdery mildew fungi

Herger (1988) proved that an aqueous extract of the leaves of R.sachalinensis induces resistance in various host plants. Effects were seen e.g. in cucumbers, tomatoes and begonias. In general the pathogens that could be controlled were powdery mildew fungi. Good efficacies were also achieved against carnation rust.
The extraction method is simple: leaves are dried and ground. An aqueous extract is prepared at a concentration of 1% and allowed to stand for one hour. The extract is filtered and applied by spraying. In order to achieve constant protection for the host plant, the extract needs to be applied every 7-10 days throughout the growing season. Application needs to be prophylactic. In greenhouse trials with cucumber reduction of mildew resulted in infections well below the economic threshold.
In experiments with begonia morphological changes in the plants were observed besides the reduction of mildew. More flowers were

built by extract treated plants and intensity of colour was enhanced. Also side shoots were reduced to a high degree. This especially is of high value for begonia growers, because normally shoots have to be removed by hand.
All these results lead to the development of a product with the trade name "Milsana" which is available on the market since 1991.
Experiments dealing with physiological changes in plants treated with R.sachalinensis extract were undertaken with cucumber plants (Schneider-Müller, 1991). It was proven that the chlorophyll content rose significantly after extract application. Enzymes playing a role in the phenolic pathway like phenylalanin- ammoniumlyase and peroxidase reached high levels only a few hours after treatment. Also the activity of ß-1,3-glucanase and chitinase increased rapidly.
Chitinase experiments were carried out with knotweed extracts from spring and summer plants. Extracts from spring plants do not reveal good protection, extracts from summer plants reach efficacies between 90% and 100%. Kowalewski (1992) observed that an increase in chitinase activity only occurred when the extract treatment resulted in a good protection against powdery mildew. In this case it could be proven that chitinase activity is highly correlated with resistance induction. The correlation coefficient is 0,96%.

2. Induced resistance against late blight of potato

A screening with extracts from more than 100 plants was carried out against P.infestans. Extracts were prepared from fresh or frozen plant material in a Soxhlett-apparatus with 96% ethanol for 4 hours. For a test of the raw extracts filter paper discs were placed in petri-dishes on rye agar as growth medium. Extract was pipetted onto the filter paper and mycelium of the fungus was placed in the middle of the dish and allowed to grow. Most extracts had no effect on P.infestans although some resulted in a slight inhibition of fungal growth. No extract could compare to the fungicide Polyram Combi.
47 extracts were further tested against P.infestans on tomato plants. After extract treatment the plants were allowed to stand for one day before inoculation. The plants were usually incubated in a slowly rotating chamber with little light where moisture was kept close to 100%. Since this "rotor" was out of order when these tests were carried out, incubation of the plants took place in an ordinary greenhouse chamber which was sprayed with water regularly to keep humidity high. All tests were evaluated when the untreated controls were infected to a high degree which was usually 5 to 7 days after inoculation. An extract was called effective when no more than 15% of the leaf area was infected.
37 of the 47 extracts were effective in both repetitions while infection of the controls was between 73% and 78%. 32 of these 37 extracts even kept infection below or at 5%!
Further repetitions were carried out with 13 extracts. The "rotor" where the tomatoes were usually incubated was working again and therefore incubation took place there. All extract treated plants showed infection rates comparable to those of the controls.
The only obvious difference between rotor and greenhouse chamber was the light condition. The rotor was fairly dark while the

greenhouse had normal daylight. To make sure that light really was the reason for those differing results the test was repeated again with 11 extracts, this time in 3 variations:

1. Incubation in the rotor
2. Incubation in the greenhouse
3. Incubation in the greenhouse under dark plastic hoods

In all variants the controls were infected from 80% to 100% while the fungicide Polyram Combi always controlled the fungus effectively with an infection rate of no more than 1%. The extract treated tomatoes in the rotor were infected between 60% and 80%, those under dark plastic hoods between 80% and 100%. In the greenhouse under normal daylight 9 out of the 11 extracts gave clear protection against P.infestans with 4 of them keeping the infection in both repetitions below 15%.
Therefore, it can be said that the tested plant extracts only give a reliable protection against P.infestans in the presence of light.
Parallel with the tests on tomato plants, where extract no. 27 was very effectiv, tests on tomato leaves were carried out in humid chambers. The ethanol was evaporated and the extracts diluted again with water to 10 different concentrations.

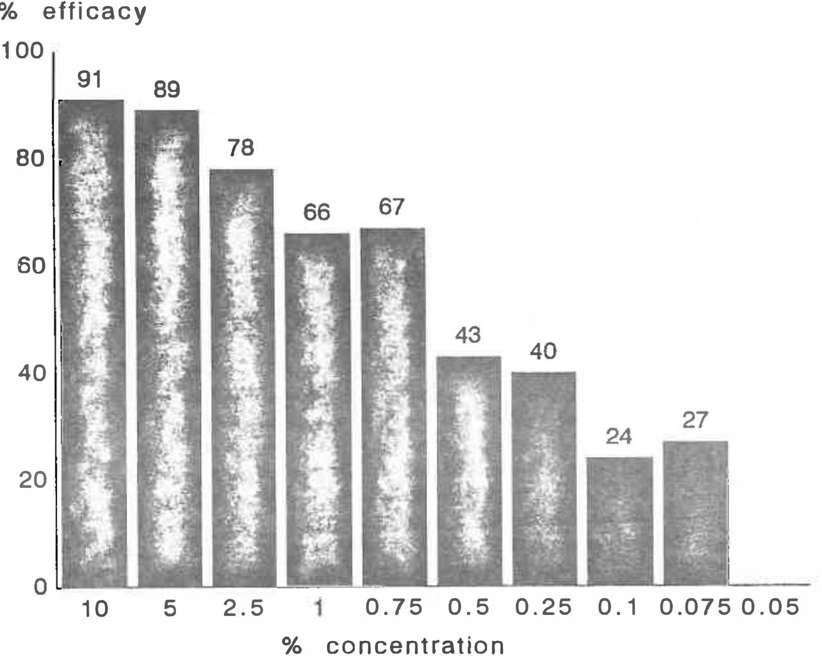

Fig.1: Efficacy of extract no. 27 tested at different concentrations against Phytophthora infestans

The test was carried out in 6 repetitions. Figure 1 shows that a concentration of 5% gives as much protection against P.infestans as 10% while lower concentrations result in less protection.
Further experiments were carried out with this extract at a concentration of 5% extracted dry matter. Application was done at daily intervals from 7 days before until 3 days after inoculation. The day the inoculation took place the extract was sprayed 4 hours before. This test was done in 2 repetitions. In addition 1 box was prepared for each day where the extract was washed off with water just before inoculation. The test was evaluated 4 days after inoculation. The results are presented in table 1.

Table 1: Efficacy of extract no. 27 at a concentration of 5% depending on time between application of extract and inoculation with Phytophthora infestans

days between appl. and inocul.	% efficacy	% efficacy (extr. washed off)
7	86	49
6	92	67
5	85	89
4	95	90
3	97	91
2	95	95
1	99	96
0	97	38
days of appl. after inocul.		
1	0	
2	0	
3	0	

Average infection of untreated controls: 79%

The extract had no curative effect while a prophylactic application gave full protection for at least 7 days. Even an application only 4 hours before inoculation was sufficient to suppress fungal growth.
Washing away the extracts before inoculation made a contact between extract and fungus impossible. Nevertheless a complete protection was assured when the extract was sprayed between 5 and 1 days before inoculation. This effect must therefore be due to an interaction between plant and extract. The lower efficacy for an application 6 and 7 days before inoculation was probably due to

the test system. After 6 respectively 7 days in the boxes those leaves had lost turgor. Some of them folded up and some caught bacteria after washing off the extract. Leaving the extract on the leaves for only 4 hours resulted in little protection against *P.infestans.*

Discussion

It could be shown that extract no. 27 had no curative effect against *P.infestans.* In the petri-dish-test no direct effect on the fungus was found. Prophylactic treatment of plants with extract no. 27 resulted in a protection of at least 7 days. The presence of light was necessary but not a contact between extract and fungus. This means that an interaction between extract and plant has been responsible for the protection against the fungus.
All this indicates that the extract induced resistance against *P.infestans* comparable to the way *R.sachalinensis* does against powdery mildew. Here also no weakening of the plants occurred.

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GENERAL DISCUSSION

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The session "Mode of action of BCA and biorational control" dealt with a range of modes of action and exemplified the points made in the opening introduction. Studies with yeast antagonists of postharvest pathogens concluded that antibiosis was not the mode of action and that more than one mechanism of action may be involved. The difficulty was to establish the precise modes of action and the details of these mechanisms *in situ* with the methods currently available. Postharvest control of *Penicillium hirsutum* rot of flower bulbs focused on the strategy of isolating bacterial antagonists from suppressive soils and showed antibiosis mainly from *Bacillus* spp. to be the mode of action *in vitro*. Whether or not this mode of antagonism will prove to be effective *in situ* requires to be established and would appear to be the next stage of the investigation before the more laborious, time-consuming and expensive steps of purification and identification of the antifungal compounds are attempted. Those antagonists which can protect *in situ* should then be selected for further study. It might even be possible to exploit these antifungal compounds for biorational control as indeed might the fatty acid induced inhibition of cutinase activity observed with the pathogens *Mycosphaerella pinodes* and *Botrytis cinerea*. This latter pathogen is also the target of study for inducing plant defence mechanisms especially with the use of avirulent mutants. It is hypothesised that the creation of a polygalacturonase-minus mutant would allow induced resistance in the host plant without disease symptoms but care will have to be taken in the creation of such a mutant to eliminate back-mutation to the virulent wild-type strain. The use of transposons and genetic manipulation might be the way forward but there is no detailed knowledge of such work with *Botrytis cinerea* and a pioneering effort would be needed here. Induced resistance need not require specific triggers and microbial metabolites from *Bacillus subtilis* can elicit resistance in barley against obligate biotrophic fungi such as powdery mildew. In these situations there may be a general overall reduction in the activity of the pathogen with reduced damage to the plant even to the extent that yield is not affected. Protection of the plant requires an interval of 48 hours and evidence is also presented that when induced resistance is raised using virulent and avirulent strains of barley powdery mildew the synthesis of new mRNA's is involved and cDNA cloning of these resistance response genes may allow their eventual role in induced resistance to be elucidated. This is an example of the use of new technology to determine the mechanisms of action of biocontrol systems. Strong evidence for the role of plant extracts of giant knotweed in induction of resistance for a range of host plants described physiological responses in enzyme activities such as increases in chitinase, ß-1-3-glucanase, peroxidase, polyphenol oxidase and phenylalanine-ammonialyase as well as morphological changes such as strengthening of papillae, to be part of the plant response in the acquisition of induced resistance. Light plays an important role here and this again suggests that there is a need to synthesise components *de novo*.

The presented data clearly indicate attempts to determine modes of action of the BCA in the development of biocontrol systems with several modes of action under exploration. Cook and Baker (1983) have stated that "in terms of the mode of action of antagonists it should be recognised that the greater the number and diversity of methods used by an antagonist to inhibit a pathogen, the more

successful it will be in biological control". It was also stated that "the greater the number of modes of antagonism a BCA has the less likely resistance will develop" (Cook & Baker, 1983). These combined benefits should not be overlooked and it may well prove useful to look for BCA's with more than one mode of action or to use more than one BCA where the individual antagonists have only one mode of action. Clearly it would be more beneficial where the modes of antagonism affect different processes important to pathogen activity (conidial germination infection, sporulation, etc.) since the antagonistic effects should then be synergistic. Equally important is that antagonists should be compatible and do not effectively cancel each other out in the attempt to control the pathogen. Given these points it could be extremely useful to explore the use of BCA combinations for effective biological control. For formulation purposes there is no reason why combinations of biorational control factors (antibiotics, fatty acids, plant extracts etc) cannot be assessed in the same way. It might be expected that the use of an antibiotic alone could result in the pathogen developing antibiotic resistance whereas its use in combination with other antifungal agents is less likely to raise resistance since simultaneous mutation to at least two parameters would be needed for this to occur. As with the use of chemical fungicides the use of BCA's and biorational controls would need to be examined and tested for toxicity, residue levels and their effect on the environment. It should however, be easier to target them to the plant surface and the infection court of the pathogen since this will be one of the strategies applied during their development for effective biocontrol. Chosen correctly BCA's should be far less damaging to the environment.

There is no doubt that with respect to the mode of action of the BCA and biorational control the underlying mechanisms of action are of fundamental importance to the future success of biological control and therefore a concerted effort should be placed on the elucidation and understanding of these mechanisms as they operate _in situ._ In the past the mode of action has often been determined and established _in vitro_ but for successful biocontrol this must also operate _in planta._ The lack of success of BCA's when applied to the _in situ_ situation has more often than not been the ecological bottle neck to implementation. The use of methodology to carry out and develop such studies _in situ_ has also been a drawback. Future research should now concentrate on the development of such methodology in order that mechanisms of action can be understood at the ecophysiological level. Once the details of these interactions become clarified so too will the necessary manipulation and modifications needed to perfect successful biocontrols. A greater understanding of modes of action of BCA's _in situ_ based on detailed studies of the interactive ecophysiology of pathogen, antagonist and host will forward their future use as environmentally acceptable disease control agents in agricultural systems.

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Commercialization of biocontrol agents

COMMERCIALIZATION OF BIOLOGICAL CONTROL AGENTS *

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Summary

The main steps involved in the process of commercialization of biocontrol agents, i.e. discovery, development and registration will be reviewed, particularly focusing on the most critical issues and major constraints to large scale implementation of their use.

1. Introduction

Biological control of plant pathogens has gained considerable attention and looks promising as a feasible complement or, less frequently, alternative to chemical control. However, although considerable research efforts have gone into the study of biological control of plant diseases over the past 30 years, to date, there is still little impact of biological products on the total pesticide market. Moreover, even in the case of the few registered BCA, their use is limited to a few, peculiar situations. A certain number of BCA is registered in North America. For instance, fungi for use against weeds, mites, pathogens are registered by EPA, as well as bacteria active against diseases or insects (Lumsden and Lewis, 1989). In Europe, where often regulatory processes do not encourage the development of BCA, only a very few of them are registered: among those, *Peniophora gigantea*, used against *Heterobasidion annosum* of conifers and *Trichoderma viride*, active against *Chondrostereum purpureum* of plum trees. Actually, to my knowledge, quite a few dossiers concerning largely tested BCA are at present waiting for registration. When the already available BCA are examined, it is evident that only very few, particularly among fungi, are active against foliar diseases, although in this field the need for alternative to chemicals is greater.
Considering the thousands of chemicals registered for use, BCA are at present insignificant components of disease management systems. However, many industry analysts and agricultural pesticide businesses agree that the transition from chemical to integrated pest control, including use of BCA, is inevitable

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* Work supported by a grant from Ministero Agricoltura e Foreste (Progetto Finalizzato: Lotta biologica e integrata. Sottoprogetto: Viticoltura).

although gradual and difficult. A Frost and Sullivam study estimates that by the end of the century, the annual Western world market for microbial pesticides will grow from $ 33-45 million to about $ 8 billion, with USA sales amounting to $ 2 billion/year (Woodhead et al., 1990). Due to the increasing cost for bringing to market new chemicals, the search for BCA could become a competitive field for the industry.

2. The major steps leading to commercialization of BCA

In the broadest sense, research toward the commercialization of a BCA includes three main steps: discovery, development and registration. Being impossible to completely cover all these aspects, only the most critical issues of each phase will be here focused.

Discovery. The market must be clearly defined before any time and money are spent in the discovery research phase. Considerable effort must be spent to determine the best proposed target (Woodhead et al., 1990). The phylosophy leading to development of BCA is somehow different in comparison with that leading to chemicals. In the case of BCA, the choice should be oriented in current production systems for which there are no economically acceptable methods of control available now or likely to be available in the future. The cost of regulatory and efficacy testing for chemicals has made some small markets - represented sometimes by one disease problem for one crop - inaccessible to chemical control. Biological control is ideally suited for use in these situations, but its development requires considerable well-financed research to produce reliable, consistent, and efficacious products (Tjamos et al., 1992). Once the possible target has been defined, the discovery research phase starts. The screening method used, which should include since the very early stage in vivo, realistic tests, is a major critical issue. Under this stage, a close cooperation between Academia and Industry is generally very beneficial. Recently, in several countries much effort, in terms of money and time, has been devoted to research in this field. However, unfortunately, too often the lack of a good planning and coordination leads to a great amount of data not always transferrable to the practice. Much more efforts should be devoted to study of mechanisms of action of BCA. This aspect, too often underestimated, is of primary importance, since the knowledge of the mode of action of BCA permits their best deployment. Moreover, the knowledge of how the microrganism and it's target interact and how the biological preparation competes in the environment will permit the increase of efficacy by allowing BCA attributes to be enhanced. It will also permit to reduce the inoculum levels, opening up a good number of markets by lowering the cost of BCA (Baker and Henis, 1990). Once, after the screening, the number of candidate BCA is sufficiently reduced to include the very best, fermentation and formulation testing can start. Also, preliminary toxicology assays, including eye and dermal irritation and

acute feeding should be initiated. If any toxicological problem is envisaged, the project should drop.
Once a BCA has shown potential for disease control based on laboratory, greenhouse and field tests, production of an effective biomass becomes a major concern. Both liquid and semi-solid fermentation systems are used for this purpose. A first step in the production of biocontrol fungi is the development of a suitable medium using inexpensive, readily available agricultural by-products with the appropriate nutrient balance. However, not always these natural substrates are consistent in composition, thus sometimes leading to major disparities in batches of similar substrates. For a successful fermentation, not only appropriate substrates are important, but also sufficient biomass containing adequate amounts of effective propagules must be obtained (Papavizas and Lewis, 1991).
The next phase is represented by formulation. A biocontrol formulation with agricultural potential should possess several desirable characteristics such as ease of preparation and application, stability, adequate shelf-life, abundant viable propagules, and reasonable cost. A good formulation can significantly increase the effectiveness of a BCA, particularly improving its survival in the environment where it is introduced, thus also its consistency in terms of control (Spurr and Knudsen, 1985). Some of the problems that BCA encounter in a hostile environment may be overcome through appropriate formulation of the product. Certain additives, such as nutrients, stickers, spreaders or carriers, can improve the effectiveness of the antagonist (Elad, 1990). The positive effect of formulation on performance of a BCA has been shown by different authors.

Development. It covers a long span of time, starting as soon as the candidate has been discovered and lasting as long as the product is commercially available. Particularly, efficacy trials must be designed under controlled and practical situation and carried out under different climatic and cultural conditions in as many locations as possible. Beside efficacy, expressed as disease control, also product viability must be very carefully monitored. This is a very delicate and critical phase. It is well recognized and it must be accepted that BCA do not often perform spectacularly and often do not work as quickly, efficiently and consistently as chemicals. At this stage of development, the choice of a candidate should be oriented to that showing a satisfactory activity under the most variable conditions. The term of comparison cannot realistically be the most active chemical but a good broad spectrum contact fungicide. Many data must be collected on compatibility of candidate BCA with other control measures and, more generally, agricultural practices. Under this phase, rates and timing of application must be defined.
Together with efficacy trials, fermentation and formulation studies continue. Also, upscaling the production is a critical step. Shelf-life of formulated BCA is a major issue related to feasibility of a biological product. Moreover, the best delivery systems must be exploited. All these aspects are

strictly related and very important in determining the success of a BCA. Also, toxicological studies must continue throughout all the development phase. Moreover, in the case of BCA, in order to protect the rights, molecular markers should be developed.

Registration. Concurrently with the developmental phase of a new BCA, research starts in order to satisfy the requirements for registration. The tests required by the different regulatory agencies, although variable in different countries, always necessitate a certain amount of time and need to be initiated as soon as possible.
These studies include: product analysis, collection of toxicological data, screening for production of known metabolites and/or toxins, residue analysis (required when the BCA will be used on edible crops or part of crops), study of ecological effects and genetic stability. Not all the protocols for the above mentioned studies have been clearly defined. Some projects, at national and EC level, are aimed at defining protocols for evaluation of BCA.
The registration process is a very crucial issue. From one side, it is clear that BCA must go through careful examination before being used under practical conditions, since "biological" does not intrinsically means safe. On the other side, the approach to biological control realistically depends on the use of large numbers of different environment-specific microorganisms: this cannot succeed with regulatory policies that require separate approval for each strain, improved strain or strain mixture and where the protocols for registration are based on those developed for chemicals (Tjamos et al., 1992). Actually, in some cases, the regulatory requirements have been designed by following the rules determined for chemicals, completely neglecting the fact that BCA are micrlandorganisms. For this reasons, BCA registration should be carried out on a case-by-case basis, by following a more flexible and tailor-made approach. This is already done, for instance, in Canada, where Industry/Academia researchers are encouraged to contact the regulators at very early stages of development in order to discuss and decide on strategies and programmes to follow (Forsyth, 1990). This approach can also make BCA development economically competitive in comparison with fungicides. In other countries, however, (i.e. most European countries) the registration process of a BCA is a complicate and slow process, with very little space for a case-by-case approach and early discussion. Moreover, a better harmonization of registration procedures, at least at EC level, is highly auspicable.

3. Conclusions

Although biological control of plant pathogens has not dramatically solved any major phytopathological problem, we are at a turning point in technology to make significant advances. It may need several years before BCA are accepted for use under a wide range of situations, but it seems likely that that time will come (Lumsden and Lewis, 1989).

Several aspects need more consideration in order to implement use of BCA. First of all, a greater cooperation between Industry and Academia is necessary. In this field of research, interchange is more essential than ever. Actually, cooperative efforts between private and public sectors are strongly encouraged at different levels, i.e. from EC: this will probably lead to practical results. A better understanding of BCA mode of action will permit to design the appropriate strategy of use and, also, the best ways of improving their activity. New approaches are necessary in order to improve production, formulation and delivery systems. Models for analysis of epidemiological, economic and biological data are necessary in order to determine maximum benefits and possible implementation into integrated disease management strategies. Moreover, efforts to develop BCA should find more support. We strongly need successful cases of BCA to be introduced into production systems to set precedents for industry and farmers to show that this approach is worthy of efforts to register and commercialize these products. Such uses could provide opportunities for small businesses or local industries, but even enterprises are likely to succeed only with assistance from public-supported institutions in registration and implementation. These successes should also demonstrate that development of BCA is economically feasible and that BCA are safe and, above all, effective. However, we must be very realistic and accept occasional failures. It is likely that BCA will rarely provide the efficacy or consistency of control that chemicals do because they are intrinsically affected more by variability in the system. However, it is worth noting that fungicides too, occasionally, can be ineffective. As use of fungicides comes under increasing criticism, the impetus will grow for use of BCA (Andrews, 1990). We can realistically hope that BCA will eventually become a significant, new component in the arsenal of tools available for IPM.

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THE REGISTRATION OF BIOLOGICAL PESTICIDES IN THE NETHERLANDS

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1. Introduction

In the Netherlands pesticides have to be registered before they may be used, stored or marketed. The Pesticides Act defines (mixtures of) chemical compounds and micro-organisms produced for the control of harmful organisms as pesticides. In this Act no distinction between chemical and biological pesticides has been made. Biological agents such as fungi, bacteria and viruses aimed for use as pesticides fall within the Pesticides Act.

2. Definition of biological pesticides

In the legislation products based on micro-organisms (fungi, bacteria) and viruses are called biological pesticides.
Nematodes, predators and insect parasites are exempted from registration. Feromones and toxines are considered as chemical pesticides.

3. History of biological pesticides in the Netherlands

In the Netherlands the first biological pesticide was registered in 1971. The active ingredient of this product was based on the bacterium *Bacillus thuringiensis* for the control of Lepidopterous larvae in cabbage, orchards and forestry. Up to now about 10 products based on *Bacillus thuringiensis* have been registered. These products are used to control Lepidopterous larvae in cabbage, orchards, forestry and fruiting vegetables and *Diptera* in caveties of houses.
This year two biological pesticides based on fungi have been registered. The first one is a product based on *Verticillium lecanii* and is used to control whitefly in cucumbers in glasshouses, the second is based on *Verticillium dahliae* used to protect elm-trees from Dutch elm disease. A few other agents have been submitted for approval.
Up till now there are no agents submitted for approval to control foliar and post-harvest diseases.

4. Registration procedure

In the Netherlands four Ministries are responsible for the registration of (biological) pesticides.

* Ministry of Agriculture, Nature Management and Fisheries
  - agricultural aspects and composition
* Ministry of Welfare, Public Health and Cultural Affairs
  - residues in food
* Ministry of Housing, Physical Planning and Environment
  - safety for the environment
* Ministry of Social Affairs and Employment
  - workers exposure

These Ministries are represented in the so-called Commission Registration Pesticides. This Commission prepares an advise on whether a pesticide can be registered or not. This advise is directed to the Minister of Agriculture, Nature Management and Fisheries who is ultimately responsible and takes the formal decision.

For the registration of biological pesticides a special questionnaire has been developed. The lay-out of the data sheets corresponds with the advice and recommendation of the Council of Europe.
This questionnaire is devided in eight chapters:
A: General information
B: Information on the use
C: Information on the formulated pesticide
D: Information on the active agent(s)
E: Information on the toxicity of the product, active agent(s) and its metabolites
F: Information on residues
G: Behaviour of the product in soil, water and air
H: Toxicity to organisms occurring in the environment

5. Evaluation of the agricultural aspects and examination of the composition

One of the main responsibilities of the Plant Protection Service (Ministry of Agriculture, Nature Management and Fisheries) is evaluating the agricultural aspects and examining the composition of agricultural pesticides.
For this purpose the information given on the application form of the chapters A, B, C and D is important.

5.1. Evaluation of the agricultural aspects

The efficacy and the agricultural side-effects of the use of a biological pesticide is evaluated on the basis of experimental data. These experimental data are obtained from field trials. These field trials are carried out by the manufacturer under the supervision of the Plant Protection Service. These trials have to be in accordance with the EPPO-guidelines for chemical pesticides or specific guidelines given by the Plant Protection Service. If possible the performance of the product is compared to the performance of a suitable reference product.
The efficacy is defined as the ability to fulfill the claims made for it on the label.
In general the following rules for efficacy holds:
- The performance of the product must be comparable to that of a suitable reference product.
- Deficiencies in the level, duration and consistency of the efficacy can be compensated by other advantages for the user.
- In the case that the use of a reference product is inappropriate or impossible the product should show a well defined benefit to the user.

The first rule is a generally accepted rule which is also used for chemical pesticides. In case the first rule is not satisfied the product can be registered when it has well-defined advantages, for instance its use in integrated control, which is often the case for biological pestices.
The rules have also been Internationally accepted. For instance they are mentioned in the draft Uniform Principles of the Council Directive of the European Community concerning the placing of pesticides on the market.
The agricultural side-effects of biological pesticides are effects on the crop (yield and quality), effects on beneficial organisms and phytosanitary risks. Clear rules for these aspects are not available so far. The performance of the product on these aspects is judged individually.

5.2. **Examination of the composition of the product**

The examination of the composition of the product is important in order to verify its efficacy after registration.

The examination of the composition consists of four aspects:

- The identity of the active agent(s)
- The content of the active agent(s) in biological active units
- The physical-chemical properties of the formulation
- The shelf-life

The identitity of the active agent(s)

Verification of the identity of the active agent(s) is to check whether the organism(s) given by the applicant is present. The methods used are based for instance on morphological and biochemical properties. Based on the information submitted on the application form the properties of the agent are examined.

The content of the active agent(s) in biological active units

Verification of the content of the active agent(s) is necessary to check whether the content given by the applicant is correct. The definition of the units in which this content is expressed is important, because the content of the product should have a relation with the activity of the product.

The method of analysis has to be submitted by the applicant and must have the possibility to standardize.

Extreme standardization namely "International Standardization" occurred in the case of products based on Bacillus thuringiensis. The content of the active ingredient is defined in International Units and also bio-assaymethods and international standard preparations have been developed.

Generally speaking every producer of biological agents uses his own method for analyzing the composition.

The physical-chemical properties of the formulation

For the use of the product the physical-chemical properties of the formulation are important. For instance a wettable powder has to be suspensable in water without clotting. Moreover, the product may not consists of too large particles causing the product to settle too quickly on the bottom of the spray tank.

Shelf-life

The shelf-life of biological pesticides is usually shorter than that of a chemical pesticide, because of the living matter of a biological pesticide. The shelf-life varies from two months at 4 C (e.g. suspensions based on fungal spores) to two years at room temperature (e.g. powders based on Bacillus thuringiensis). It is important for the efficacy of the product that during the maximum shelf-life period there occurs no loss of biological activity.

6. Policy on pesticides

The Dutch policy on biological control is laid down in the Multi-Year Crop Protection Plan. The most important target of this Plan is to reduce the dependence on pesticides, this establish a reduction in the use of chemical pesticides. One of the solutions given is to encourage biological control. This stimulation of biological control goes by the way of the reseach on biological control and by the way of the extension to the farmers to use biological methods. Also the agricultural industry is encouraging growers change to biological methods. For instance the Central Bureau for auction of horticultural products designed requirements for

growing tomatoes and cucumbers. One of these requirements is that several pests have to be managed by means of biological control methods such as predatory mites for the control of spidermites. The products are given a special label (butterfly) to draw consumers' attention on these environmentally safe grown products.

7. <u>Problems concerning the registration of biological pesticides</u>

The existing problem with respect to the registration of biological pesticides is a result of mainly unclear requirements for toxicological data, which are often very expensive. In general there is little experience with the registration of biological pesticides and there is a lack of requirements for the required data for the registration. Because of this obscurity the producers of biological pesticides have no view on the costs to have their products registered. Another point is that most biological pesticides have a small area of use. Because of this specific efficacy of the agent the costs/profits ratio for the development of a biological pesticide is often unacceptable.
One of the improvements for the registration procedure is the publication of the earlier mentioned questionnaire for the registration of biological pesticides, in which requirements have been formulated.

8. <u>Future developments</u>

In 1991 the Council Directive of the European Community concerning the placing of pesticides on the market has been adopted. The purpose of these Directive is to achieve an harmonized system for the registration of pesticides within the European Community. In one of the Annexes of the Directive a list of data requirements for the file needed for the registration of a plant protection product has been included. For biological pesticides there has been a special list added which differs from the one for chemical pesticides. At this moment the explanation on the data requirements is under discussion so that there is no view on the extend of the requirements for biological pesticides.
By harmonization the required data for the registration can be used in all countries of the European Community. This will increase the possibilities for the producers of biological pesticides to place these products on the market.

From this review it appears that the registration procedure of biological pesticides is a developing process. Nowadays a lot of research on biological control agents takes place. It is expected that an increasing number of agents will be developed to biological pesticides.
To stimulate the development, registration and use of biological pesticides good cooperation between research, industry and authorities is necessary.

IMPLEMENTATION OF BIOLOGICAL CONTROL OF FOLIAR DISEASES

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Summary
The initial conditions for the development of biocontrol products stipulate the existence of a good potential biocontrol agent, a niche for commercial exploitation, reliability of the preparation, patentability, registerability, field effectiveness, and nontoxicity. These aspects are discussed. The preparation of *Trichoderma harzianum* T39 'Trichodex' for biocontrol of grey mould serves as an example for successful biocontrol preparation.

Introduction

Biological control of foliar pathogens has been studied for many years, but the number of biocontrol preparations available on the market for grower use is very small (Elad, 1990; Lynch, 1988; Powell, 1992). Nowadays, national authorities have become increasingly aware of the drawback of using many chemical pesticides, in terms of their impact on the environment, growers, and consumers of agricultural products. Research aimed at replacing pesticides by environmentally - safer methods is currently underway at many research centres. Biological control is a potentially viable means of controlling disease. At present, we are witnessing intensive activity towards the market introduction of an increasing number of biocontrol agents.
The organisms involved in disease control include the pathogen and other microorganisms, the host plant, and the antagonist. Each of these are affected by one another and by the environment, by cultural practices, and by pest control programs. Indeed, the phylloplane microflora are characterized by their diversity and complexity. The biological components of the plant surfaces are influenced by exudates, fluctuating temperatures, relative humidity and free water, atmospheric gases, light and radiation, wind, and pollution. Factors tend to change radically at all times.
It is obvious that a biocontrol microorganism will not persist and remain active unless it is adapted to the plant environment. It must compete with other microorganisms and establish an active population on the phylloplane. An introduced antagonist should, above all, interact efficiently with the pathogenic invaders.
There are several biocontrol agents, that are reportedly as effective as fungicide control (Mukerji & Garg, 1988); however, in most cases, the results obtained for many agents are mediocre and inconsistent. Nevertheless, it seems that a moderately - effective but consistent agent suffices to establish non-chemical control of plant disease and reduce the level of pesticide residues in agricultural products. The introduction of such an agent calls for a major change in quality grading of agricultural produce, and education of the customers. Successful control by biological means on the phylloplane, that has been reported in literature, involves mainly rusts and powdery mildews, and diseases caused by the following genera of pathogens: *Alternaria, Epicoccum, Sclerotinia, Septoria, Drechslera, Venturia, Plasmopara, Erwinia,* and *Pseudomonas*. Hence, we should expect to see in the future market biocontrol preparations against these pathogens and others.
The initial conditions for the development of biocontrol products stipulate the existence of a good potential biocontrol agent, a niche for commercial exploitation, reliability of the preparation, patentability, registerability, field effectiveness, and nontoxicity. These aspects will be discussed further below. Insufficient research efforts have been directed towards the selection of characteristics that enhance the survival of biological control agents; however, several techniques are available that were developed by microbial ecologists and the fermentation industry to select for survival and manipulation of beneficial microorganisms under given environmental conditions, including temperature,

osmotic pressure, radiant flux, pH, and nutritional level. Moreover, formulation of the biocontrol product can supply us with a preparation that has a long shelf life, enabling it to withstand adverse conditions and even supply it with the right ingredients to induce its specific activity.

Selection of biocontrol agents

Mechanisms other than those measurable in petri dishes are of importance under field conditions. Among these are probably the ability to live and grow on the plant surface under different nutritional and microclimatic conditions, and to colonize the plant in such a way as to prevent the establishment of the pathogen. This makes it very difficult to select antagonists for field trials, and the problem is complicated further by virtue of the fact that an isolate - although effective against a pathogen on one crop - may be ineffective against the same pathogen on another crop. The use of adequate selection methods is probably the main key to a successful biocontrol system. Selected isolates of potential antagonists should originate from the habitat in which they are designated for use (i.e.) (similar environment conditions, cropping system, growth, amount of nutrients, diseased organs, and pathogenic flora).
In order to select microorganisms under conditions as close as possible to those *in vivo* , we have selected antagonists to *B. cinerea* by applying their suspensions on detached leaves of susceptible hosts, subsequently inoculating them with the pathogen. This procedure was repeated several times, because the results were not usually consistent. The amount of nutrients in the bioassay system should also be taken into account, because too high a level of nutrients might mask the effect of competitors. The ultimate test in the screening procedure is the performance of the prospective antagonist when tested under natural conditions with the variable microbial community of microorganisms, and when conditions are unfavourable.

Formulation and delivery system

A biocontrol agent, that has been proven effective under controlled conditions or in the field, is only one component of a successful product. In order to develop a preparation that will meet market requirements it should be formulated. The history of *Trichoderma* development for biocontrol can supply us with a good example. This biocontrol agent was grown in various laboratories on wheat bran with or without peat, on molasses-enriched clay granules or seeds Backman, 1975; Hadar et al., 1979; Sevan et al., 1984; Huang et al., 1985. Papavizas et al. (1984) reported performing large batch production of *Gliocladium, Talaromyces*, and *Trichoderma* spp. by liquid fermentation, utilizing commercially - available ingredients that consisted of molasses plus either dried brewers yeast, cotton seed flour, or corn-steeped liquor. Recently, a product for foliar use of *Trichoderma* (T39) was developed in Israel. This preparation ("Trichodex" produced by Makhteshim) is a wettable powder of the fungus, that contains 10^{10} CFU per gram and is designed to control grey mould of various crops.
"Trichodex" has a good shelf life and its formulation enables it to survive well on the canopy (Elad, 1992; Elad et al., 1992). The preparation performs consistently in controlling the disease, better than a non-formulated culture of the same isolate.
Yeast, or bacterial biomass from liquid fermentation, has commercial potential (Wilson & Pusey, 1985. Wilson & Wisniewski, 1989). A similar system using a pruning apparatus involves the application of a spore suspension of *Trichoderma* to trees to prevent silver leaf caused by *Chondrostereum purpureum* (Dubos, 1987). A streptomycete formulation called Mycostop™ is also commercially - available in Europe as a wettable powder (Tahvonen, 1986). The preparation is reportedly effective against various diseases, including lettuce grey mould.
Wet fermentor biomass of fungi or bacteria, or the dry powdered biomass, may be used as biocontrol agents. Use of wet biomass or whole suspension is advantageous

because it omits a drying and milling step, and enables easy processing of some fungi whose biomass cannot be separated readily from spent liquid because of gum formation. For some biocontrol agents, solid state fermentation may suffice, as mentioned above for *Trichoderma*.

In their comprehensive report on formulations, Connick et al. (1990) concluded that an appropriate forumulation can help tip the host-pathogen balance to favour activity by the biological control agent. I agree that it has the most pronounced influence on biocontrol activity under commercial conditions. The formulation must be compatible with the requirements of the microorganism, even enhancing its performance, and should be compatible with regular agricultural practices. In order to enable distribution throughout the normal commercial chain, the preparation should have a shelf life as long as 1-2 years; however, this is not expected to be the real case for the common biocontrol preparation. In order to protect the microorganism, the formulation should be able to induce low metabolic activity in the microorganism involved. This is usually achieved by reducing moisture potential by drying or by incorporating the agent into an oily microaerophilic environment. Surfactant, stickers, emulsifiers, and spreaders are added to formulations in order to improve their application to the target. The harmful effect of irradiation on the foliage is usually reduced by sunsceening compounds or microencapsulation (Rhodes 1990). Powell (1992) suggested screening the agents not only according to their disease control capability, but also according to their suitability for production, as this will be critical to control in an economic process of cost-effectiveness for the farmer.

Improved efficacy

An approach of combining the biocontrol agent with fungicides was exemplified in the system of grey mould. "Trichodex" was tested under commercial conditions in vegetable greenhouses and grape vineyards: sprayed alone, in tank mix with iprodione, or alternated with iprodione or diethofencarb + carbendazim. Up to 84 % disease reduction was obtained with the biocontrol agent, that was at least as effective as iprodione or vinclozolin. The tank mix of *T. harzianum* with iprodione tended to be more effective than either of the agents alone (Elad & Zimand, 1991, 1992). Alternating the biocontrol agent with fungicide was also effective. Thus, is possible to reduce the exposure of *B. cinerea* populations to fungicides, reduce fungicide use, and potentially minimize pesticide residues in the treated agricultural products (Elad, 1992; Elad et al., 1992).

Another suggested arena for activity is the production of inoculum by the pathogen. This approach is common among biotrophic plant pathogens such as powdery mildews and rusts. Fokkema et al. (1992) studied it also with necrotrophs such as *Sclerotinia sclerotiorum* and *Botrytis spp.*, suggesting the reduction of sclerotial production of the former pathogen or of conidia of the later pathogen in a standing crop so that a later crop would be faced with reduced amounts of inoculum of pathogens. A broader approch should be pursued, whereby biocontrol agents will be applied in order to prevent inoculum production, its survival, and its ability to infect host tissues. In order to achieve this approach, a combination of micro-organisms may be used effectively in one or more stages of the disease cycle.

Although a large variety of effective control agents can be isolated from natural habitats, genetic modification of these microorganisms may result in the improvement or acquisition of certain traits important to biocontrol activity. One of the simplest methods evaluated for improving biocontrol agents is mutation by chemical mutagens or irradiation. This method has already rendered in mutants resistant to fungicides and featuring various enhanced characterizations of growth, sporulation and survival (Papavizas, 1987). Recombinant DNA techniques and protoplast fusion may also supply new biotypes of biocontrol agents (Statz et al., 1989; Shapire et al., 1989).

Commercialization

Registration entails a long complex procedure to prove that the preparation is not dangerous to animal and human health or the environment, and that it is a reliable product from an agricultural point of view. In many countries, this registration involves several different authorities e.g., in the area of agriculture, health, and environment, that are not easy to coordinate.

Patenting of a preparation, the biocontrol agent itself or its use is one of the key issues facing the producers of biocontrol agents today. Patenting of the microorganism itself may not be feasible, as the authorities may be unwilling to patent naturally - occurring microorganisms. Therefore, there is now a tendency to patent the process of production or the use of a preparation, that can give the manufacturer more protection. There are many plant diseases important in current production systems for which there are no economically or environmentally - acceptable methods of control available at present or likely to be present in the future. Moreover, several small-market-diseases are inaccessible to chemical control because the cost of regulatory and efficacy testing for chemicals has made it very expensive. Biological control is suitable for such market niches. To ensure its reliability, consistancy, and efficacy, well-financed research is needed. It can be assumed that the number of different strains of biological control agents or formulated products needed to control the many plant diseases will be large, owing to the diversity of crops, and the wide range of environment conditions. The current regulatory policies that stipulate a separate approval for each strain, improved strain or strain mixture (as is required of chemical products) impose a major burden on producers of biocontrol produces, and may discourage them from developing variable products.

The public and the regulatory agencies are concerned about environmental and human hazards that may appear once a new biocontrol agent is released onto the market. However, it should be noted that for seven decades of work with introduced (non-engineered) micro-organisms, so far no experimental or commercial use has resulted in a new disease problem. This is because the biocontrol agents are usually reintroduced to augment their populations in a similar habitat from which they were isolated and where they occur naturally. This is similar to agricultural practices such as crop rotation, soil tilling or soil enrichment with green manure change the microflora and new ecological balance in a microbial population. The biocontrol process has the potential to solve agricultural problems; therefore, it should be developed further by research institutes and commercial enterprises for the benefit of the farmers.

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INVESTIGATIONS ON PREVENTION OF STRAWBERRY GREY MOULD (*BOTRYTIS CINEREA* PERS.) IN ROMANIA BY USING THE FUNGUS *TRICHODERMA VIRIDE* PERS. *EX* S.F. GRAY

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Summary

Out of 14 isolates belonging to 6 species of saprophytic fungi tested *in vitro* for assessing their antagonism to *Botrytis cinerea* in strawberry, 6 isolates of *Trichoderma viride* proved their antagonism. The bioproducts based on *T. viride* dry biomass (Td_5, Td_{23}, Td_{49}, Td_{50}) used alone, showed good effectiveness in preventing grey mould on cv. Senga Sengana under field conditions, high yields of healthy fruits being thus obtained with low *B. cinerea* attack frequency.

Introduction

Grey mould induced by *Botrytis cinerea* is one of the most damaging diseases of strawberries, its control using prevalently up to present chemical means, these leading to accumulation of fungicide residues in fruits. To avoid these, recent research was pointed to non-chemical, unpolluting ways for its control, among which the biological ones having an outstanding share (Tronsmo & Dennis, 1977; Tronsmo, 1981, 1986; Gullino *et al.*, 1985; Peng & Sutton, 1990; Sutton, 1990). Such research also started in Romania, primarily directed to the use if antagonistic fungi (Şesan & Teodorescu, 1987; Teodorescu & Şesan, 1988).

Materials and methods

The following biological materials have been used: one *B. cinerea* isolate ($B.c._1$) obtained at I.C.P.P. Maracineni-Arges, 14 isolates of saprophytic fungi belonging to 6 species and 15 *T. viride* mutants (Td_A...Td_O) obtained at I.C.P.P. Bucharest.

In order to reveal the *in vitro* relationships between *B. cinerea*, on one hand, and the isolates of saprophytic fungi and *T. viride* mutans, on the other hand, the method of double cultures has been used (Şesan & Teodorescu, 1987).

Field tests were performed at I.C.P.P. Mărăcineni-Arges in 1986-1990 with *T. viride* isolates (dry biomass) on cv Senga Sengana, highly sensitive to grey mould. Four treatments have been applied on warning in the stages: green and white flower-bud, start of petal fall and fruit formation, the applications being combined with insecticides such as Thiodan R 35 and Zolone 35 EC, both at 0.2% rates.

Scorings were effected by using the attack level on fruits and yield, compared to a standard (Rovral 50 WP) and an untreated check. Efficacy of treatments was calculated with Abbott's formula.

Test plots covered 15 m^2; the variants were randomized with 3 replications. Data processing used the Duncan-test.

Results and discussion

Among the 14 saprophyte fungi isolates tested *in vitro* vs. B.c.$_1$ only those of *T. viride* were antagonistic (Fig.1). The isolates from *Verticillium tenerum* (V.t.), *Gliocladium roseum* (Gl), *Trichothecium roseum* (Tt), *Sordaria fimicola* (Sprd.) and *Chaetomium* spp. (Ch.) were not active against B.c.$_1$.
The 15 *T. viride* mutants acted more intensely antagonistic, as compared to Td$_5$ isolate which they derived from, the most active being Td$_D$, Td$_H$, Td$_I$, Td$_K$, Td$_N$ and Td$_O$.

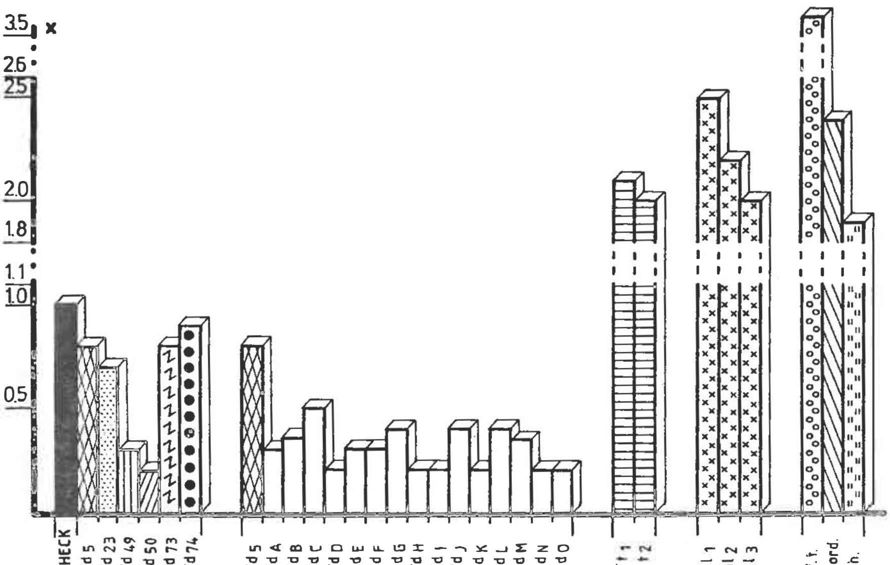

Figure 1. "In vitro" antagonism of some saprophytic fungus isolates and **Trichoderma viride** mutants versus **Botrytis cinerea** on strawberries as expressed by x coefficient

Field results are illustrated by the data obtained in 1986 (Table 1), when climate conditions were highly favourable to a severe outbreak by grey mould (AL = 37.4%).
In test-variants the attack level (AL) by grey mould ranged between 13.1 and 19.7%, the lowest being in the variant with Td$_{23}$ (13.1%), followed by Td$_5$ (14.4%), Td$_{49}$ (16.6%), while the highest was recorded in Td$_{50}$ (19.7%). In all cases these values overpassed the standard (Rovral 50 WP), where AL was 9.8%, the compound used being a specific highly effective antibotrytis fungicide. Treatments with *T. viride* provided a suitable protection of strawberries against the grey mould, its efficacy ranging between 65.0 and 47.3%, as compared to the standard (73.8%). The highest efficacy (E) was noted with the isolate Td$_{23}$ (65.0%), followed by Td$_5$ (61.5%) and the lowest in Td$_{49}$ (55.6%).
Effectiveness in controlling *B. cinerea* (CE) by the isolates of *T. viride* fluctuated within the limits 88.1 and 64.1%, as compared to the chemical standard (100%). These results are also reflected by the yield resulted, being comprised between 4.1 and 3.7 kg / 15 m^2, as compared to 4.3 kg in the standard and 3.6 kg obtained in the untreated check.

Table 1 Efficacy of treatments with *Trichoderma viride* for preventing grey mould of strawberries

Variant	Conc. (%)	AL (%)	E (%)	CE (%)	Fruit yield (kg / 15 m^2)
Td_5	0.2	14.4	61.5	83.3	4.0
Td_{23}	0.2	13.1	65.0	88.1	4.1
Td_{49}	0.2	16.6	55.6	75.3	3.8
Td_{50}	0.2	19.7	47.3	64.1	3.7
Rovral 50 WP (standard)	0.1	9.8	73.8	100.0	4.3
Check	-	37.4	-	-	3.6
LD 5%		11.6			

Yields of variants with biological treatments, though lower than for chemical applications, are nevertheless close in some cases (Td_{23}, Td_5) to this, taking the advantage of being devoid of fungicide residues.
The technology of strawberry protection should take into account a wide range of morpho-physiological factors of this plant, on which meteorological and pedoclimate factors act (Fig. 2), primarily influencing photosynthesis and, implicity, distribution and redistribution of assimilates, these processes leading to formation of vegetative organs, flowers and fruits. The same factors also influence the pathogenic flora of plant, and also the beneficial one. The study of interactions between the pathogenic and beneficial flora is revealed as an outstanding way to knowledge and intervention for a benefic management of useful microorganisms populations, among which those antagonistic to the main pathogens.

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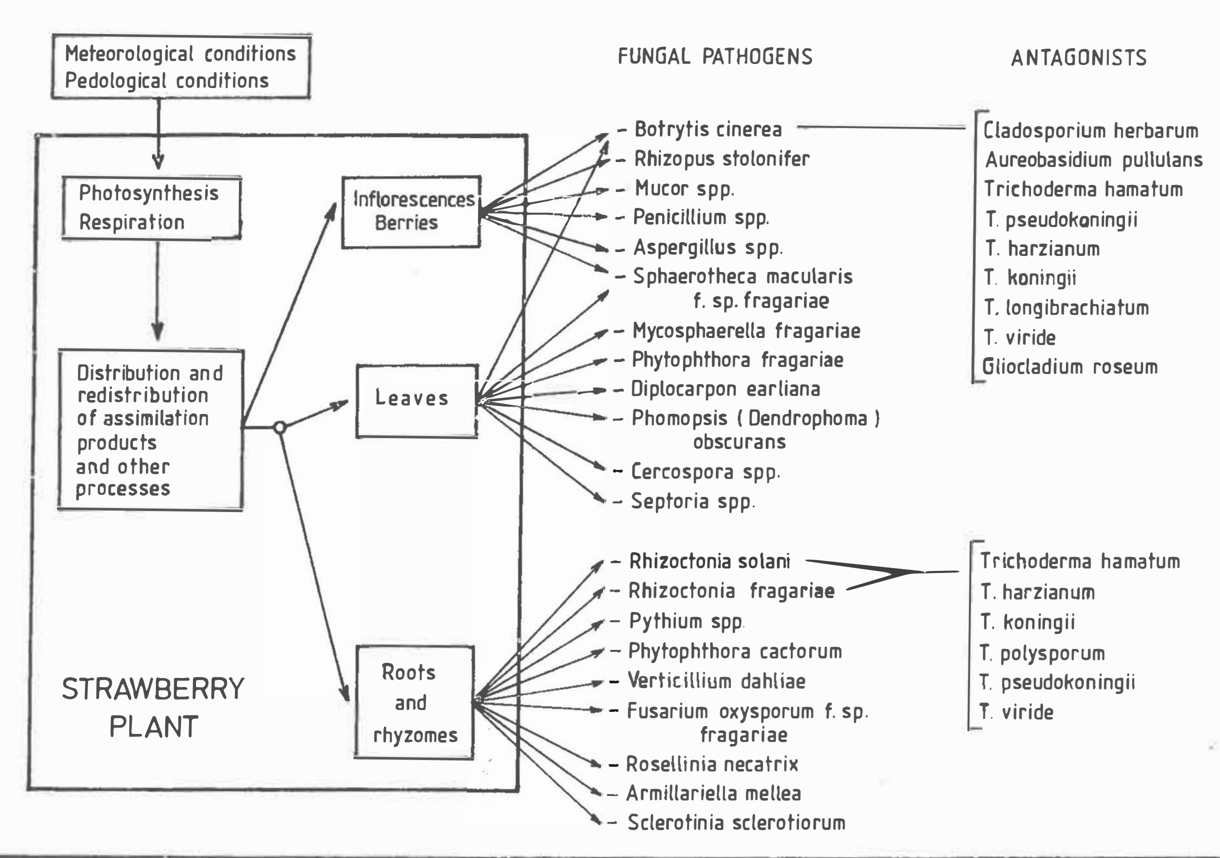
CONCEPTUAL PATTERN OF STRAWBERRY PLANT – PATHOGENS – ANTAGONISTS SYSTEM (ŞESAN – 1992)



INVESTIGATIONS ON PREVENTION OF GRAPEVINE GREY MOULD (*BOTRYTIS CINEREA* PERS.) IN ROMANIA BY USING THE FUNGUS *TRICHODERMA VIRIDE* PERS. *EX* S.F. GRAY

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Summary

Bioproducts containing dry biomass of an isolate of the antagonistic fungus *Trichoderma viride* (Td_S) and mutants Td_E, Td_M, Td_K and Td_N were effective when exclusively used in field to prevent grey mould and enabled to obtain high grape yields.

Introduction

The grey mould of grapevine, one of the key diseases with high economic significance (fig. 1) caused always a great concern for scientific research, aiming at its efficient control. Besides the use of chemicals, during the last two decades studies on the biocontrol of this disease were also approached, referring particularly to antagonistic fungi from *Trichoderma* genus in France, Italy and other geographical areas among which in Romania (Mirică et al., 1990; Şesan, 1990; Şesan & Podosu, 1990).

Materials and methods

Grapevine cv Tămîioasă Românesască was used, and bioproducts from dried biomass of the isolates Td_S, and 7 *Trichoderma viride* mutants (Td_C, Td_E, Td_I, Td_J, Td_K, Td_M, Td_N), obtained and selected at I.C.P.P. Bucureşti (Şesan & Baicu, 1989), the experiments being set up at S.C.P.V.V. Odobeşti-Vrancea as latin rectangle, in variants with 3 replications, enclosing 20 plants each. Treatments were applied on warning, with some 1,000 l/ha in the phases: after petal fall, at the beginning of berries formation, at the start of ripening and 3 weeks before harvest. To establish efficacy of applications we have determined: the attack frequency and intensity on leaves and grapes, average grape yield per stump and sugar content (g/l) in must. The attack degree was calculated with the attack frequency and intensity. Efficacy of biological treatments was calculated with Abbott's formula, and the control effect was evaluated in comparison with the chemical standard - Sumilex 50 WP. Data were processed as analysis of variance.

Results and discussion

The results are presented in tables 1 and 2, including data from 1988 and 1989, years favourable to grey mould infections. In 1988 efficacy of bioproduct treatments was 81-98%, as compared to the chemical application, where efficacy (E) was 94%. The best control effect (CE) was noted in treatments with dry biomass of the mutants Td_E (104.2%), Td_M (103.2%), Td_K and Td_N (102.1%), which overpassed the chemical standard Sumilex 50 WP. These results are confirmed by the data referring to grape yield/stump and sugar content (table 1).

Table 1 Efficacy of treatments with *Trichoderma viride* mutants for preventing grey mould in grapevine in 1988

Variant	Conc. (%)	AL (%)	E (%)	CE (%)	Yield (kg/stu-mp)	Sugar (g/l)
Td_5	0.2	3.3^{xxx}	98	104.2	11.5^{xxx}	170.9^{xx}
Td_C	0.2	1.7^{xxx}	90	95.7	11.1^{xx}	167.5
Td_E	0.2	0.4^{xxx}	98	104.2	10.0	166.4
Td_I	0.2	3.4^{xxx}	81	86.2	10.8^{xx}	181.5^{xxx}
Td_J	0.2	2.7^{xxx}	85	90.4	12.4^{xxx}	163.5
Td_K	0.2	0.7^{xxx}	96	102.1	12.5^{xxx}	198.3^{xxx}
Td_M	0.2	0.5^{xxx}	97	103.2	12.1^{xxx}	174.2^{xxx}
Td_N	0.2	0.6^{xxx}	96	102.1	10.1	178.4^{xxx}
Sumilex	0.2	1.0^{xxx}	94	100.0	12.7^{xxx}	166.4
Check	-	17.6	-	-	9.6	161.6
	LD 5%	0.4			0.9	6.2
	LD 1%	0.5			1.2	9.3
	LD 0.1%	0.7			1.6	10.9

Efficacy of bioproduct treatments in 1989, more favourable to grey mould attack (AL = 29.2% in check), was 91.1 - 95.5%, higher than the chemical standard (88.0%).
Grape yield/stump and sugar content in must were by far higher than in the untreated plot (table 2).
All data collected revealed a good efficacy of treatments with *T. viride* bioproducts, comparable to that of the classic chemical treatment. The bioproducts, from both Td_5 isolate and mutants of this, resistant to TMTD and methyl thiophanate, especially Td_C, Td_E, Td_M, Td_K and Td_N, had control effects by 2.1 - 8.0% higher than the chemical standard Sumilex 50 WP. At the same time, grape yield and its quality, as reflected by the sugar content, were higher in the treated plots than in untreated ones. Treatments with bioproducts present the advantage of being non-pollutents to soil and grape harvest, therefore introduction of biological measures in grapevine protection is a beneficial chain loop for plant and man.
Introduction of biological means for grapevine protection should start with knowledge of the whole morpho-physiological complex of plant growth and development, of actions of meteorological and pedoclimatic factors, of all elements interferring in the grapevine agroecosystem, among which the "principal" and "secondary" harmful organisms. Among the principal ones *Botrytis cinerea* is also included, however this pathogen could have an overwhelming significance in dependence of the above mentioned conditions, while during the years when unfavourable conditions prevail no control interventions are necessary.

Table 2 Efficacy of treatments with *Trichoderma viride* mutants for preventing grey mould in grapevine in 1989

Variant	Conc. (%)	AL (%)	E (%)	CE (%)	Yield (kg/stump)	Sugar (g/l)
Td_5	0.2	3.3^{xxx}	98.0	101	4.4^{xxx}	196.5^{x}
Td_C	0.2	1.3^{xxx}	95.5	108	4.7^{xxx}	193.0
Td_I	0.2	2.3^{xxx}	91.8	105	4.6^{xxx}	197.5^{x}
Td_M	0.2	2.5^{xxx}	91.4	104	4.6^{xxx}	225.8^{xxx}
Sumilex	0.1	3.5^{xxx}	88.0	100	4.4^{xxx}	219.8^{xx}
Check	-	29.2	-	-	3.3	165.6
	LD 5%	2.1			0.5	30.3
	LD 1%	2.9			0.6	41.9
	LD 0.1%	3.9			0.9	57.9

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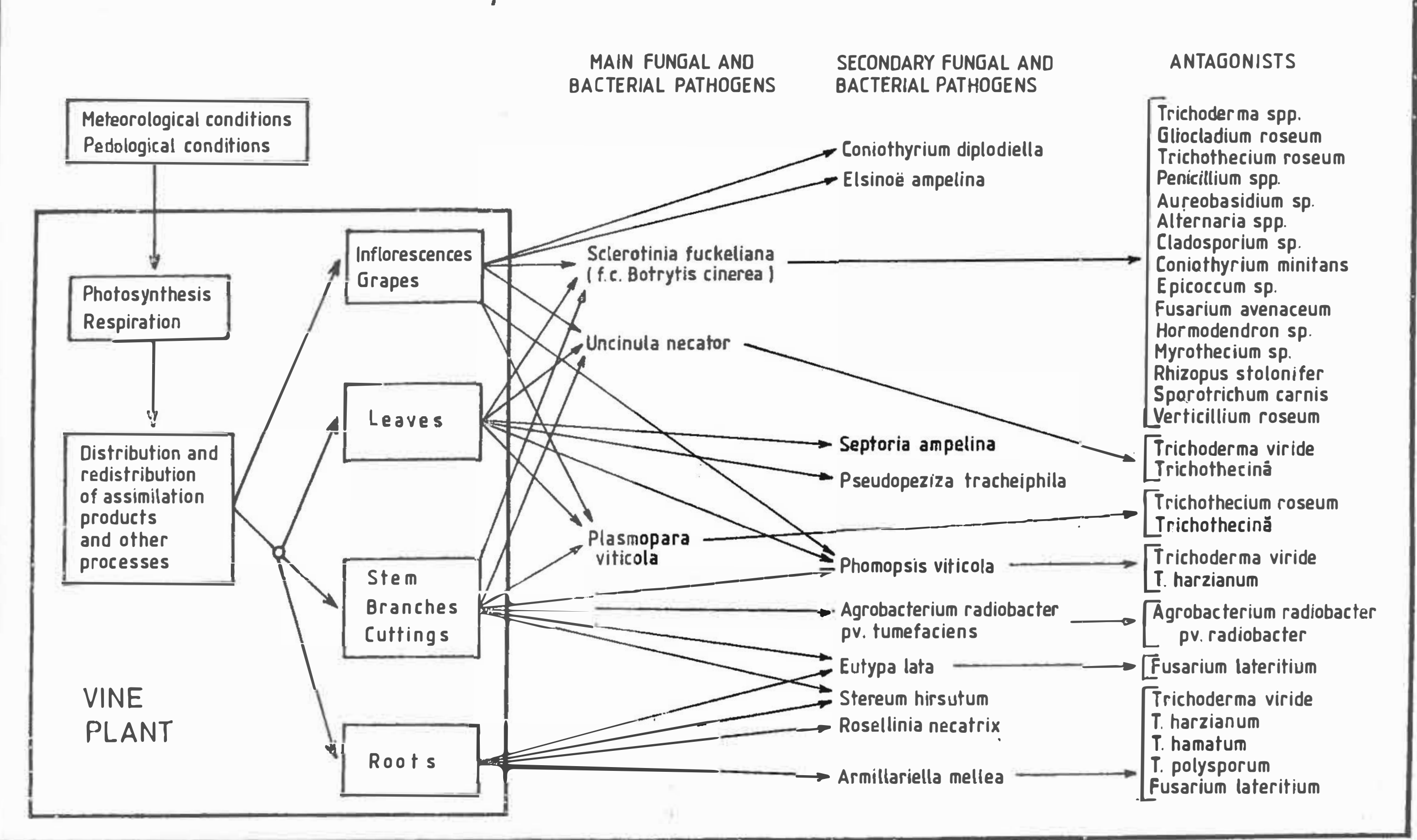
CONCEPTUAL PATTERN OF VINE PLANT – PATHOGENS – ANTAGONISTS SYSTEM
(ȘESAN – 1992)
MAIN FUNGAL AND BACTERIAL PATHOGENS
SECONDARY FUNGAL AND BACTERIAL PATHOGENS
ANTAGONISTS
Meteorological conditions
Pedological conditions
Photosynthesis
Respiration
Distribution and redistribution of assimilation products and other processes
VINE PLANT
Inflorescences
Grapes
Leaves
Stem
Branches
Cuttings
Roots
Sclerotinia fuckeliana
(f.c. Botrytis cinerea)
Uncinula necator
Plasmopara viticola
Coniothyrium diplodiella
Elsinoë ampelina
Septoria ampelina
Pseudopeziza tracheiphila
Phomopsis viticola
Agrobacterium radiobacter pv. tumefaciens
Eutypa lata
Stereum hirsutum
Rosellinia necatrix
Armillariella mellea
Trichoderma spp.
Gliocladium roseum
Trichothecium roseum
Penicillium spp.
Aureobasidium sp.
Alternaria spp.
Cladosporium sp.
Coniothyrium minitans
Epicoccum sp.
Fusarium avenaceum
Hormodendron sp.
Myrothecium sp.
Rhizopus stolonifer
Sporotrichum carnis
Verticillium roseum
Trichoderma viride
Trichothecină
Trichothecium roseum
Trichothecină
Trichoderma viride
T. harzianum
Agrobacterium radiobacter
pv. radiobacter
Fusarium lateritium
Trichoderma viride
T. harzianum
T. hamatum
T. polysporum
Fusarium lateritium

COMMERCIALIZATION OF A BIOCONTROL AGENT

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Introduction

In the Netherlands most of the pests in glasshouse fruit vegetables are being controlled biologically. Diseases however, are still controlled by chemical products. An important disease is powdery mildew, especially in cucumber. Cucumber growers spray their crop every 10 to 14 days against powdery mildew (*Spaerotheca fuliginea*) with a total of about 20 times a year. Also in tomato and sweet pepper powdery mildew is a problem. Powdery mildew can be effectively controlled by chemical fungicides. A new concept is the biological control of this disease.

On this moment Koppert is investigating the possibilities of developing a biological fungicide against powdery mildew in glasshouse vegetables. Since Koppert is mainly working in the biological control of arthropodal pests, the biological control of fungal diseases is a new topic for us.

Why are we interested in the biological control of powdery mildew ?

Till now, there are no resistant or only partly resistant cultivars against powdery mildew in use in glasshouse vegetables. Moreover, the use of fungicides against powdery mildew has some shortcomings. Fungicides can have a side effect on beneficials, such as natural enemies and pollinators. For example, pyrazophos (Curamil) is harmful for all natural enemies and pollinators; triforine (Funginex) is harmful for predatory mites. Fungicides can have a fytotoxic effect on the crop, by crop damage and growth inhibition. Fungicides can lose their efficacy, due to resistance of powdery mildew against these products. Therefore, fungicides out of different chemical groups have to be used alternately, to prevent this resistance problem. The use of fungicides will probably be reduced for environmental and other reasons. In future some fungicides may be even restricted by the government.

What are the requirements for such a biofungicide ?

The biofungicide should be effective in different crops and at the standard climatic conditions. The efficacy should be comparable with the existing chemical control. If possible, it should have a preventive as well as a curative effect. The price of the product should be competitive with the existing chemical control. Moreover, the growers will prefer a handeable product, which is easy to store, suitable for the existing spray equipment and compatible with controls of other pests and diseases. Furthermore the product should be harmless to beneficials and safe for man and environment.

What sort of research is needed ?

In general, research at our company is focussed on the evaluation of new biocontrol agents, followed by the development of promising biocontrol agents into marketable products. During the process of product development many research steps have to be taken.

Research concerning the biocontrol of powdery mildew is directed on searching and screening for effective antagonists and formulations, on spore production, downstream processing (harvesting, drying and formulating), storage and testing in horticultural practice. If this research results in an effective and producible product, the registration procedure can be started.

At this moment, we are still in the initial phase of this project. We have found some promising isolates and formulations and we are investigating a suitable production method.

What are the bottle-necks in this research ?

For the screening research it is necessary to find a suitable and standardized bioassay. Since powdery mildew is an obligate pathogen, the use of fresh plant material will be essential in this screening method. The effectivity of the antagonists is very dependent on the crop, climatic conditions and formulation of the product. Therefore these factors has to be standardized and adjusted to the practical situation. Also for the quality control of the product we need a standardized bioassay, with emphasis on a small-scaled, short-termed and easy-to-replicate method.

Little knowledge exists on production, formulation and storage of microbials. Factors controlling these processes are not yet understood. Just by the experience we have with other microbial products and a little trial and error, we try to find suitable methods for this.

The registration of the product is a very expensive and time-consuming procedure. Financially the registration procedure is a very uncertain factor. Until now, there exists uncertainty about which toxicity research has to be carried out. In first instance acute toxicity tests have to be done, with the possibility that sub-acute and even chronic tests have to be performed at a later stage. It depends on the amount of toxicological research that has to be carried out, if obtaining a registration will be economically feasible. Concerning the period of time, the registration with the authorities takes a minimum of two years.

Discussion

In general, research at universities and institutes results in new ideas on biological control methods. If an idea is to be developed into a product by a commercial company more research is needed to bring this biocontrol agent finally on the market. The question is which research has to be carried out by universities and institutes, and which by the commercial companies. Important is that with a closer cooperation between both sides results may be obtained faster and overlapping research may be prevented. Beside the cooperation between plant pathologists, also the knowlegde of other disciplines (for example microbiology or process technology) may be essential to get a biocontrol agent on the market.

USE OF BEES TO DELIVER BIOCONTROL AGENTS FOR CONTROLLING FLOWER-INFECTING PATHOGENS.

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Summary
An overview is presented of recent investigations on the vectoring of microbial biocontrol agents by bees to flowers of several fruit crops. Spores of the fungal antagonist *Gliocladium roseum*, formulated with talc and cornmeal, were vectored to flowers of strawberry and raspberry, and effectively suppressed fruit rot caused by *Botrytis cinerea*. The bacterial antagonists *Pseudomonas fluorescens* and *Erwinia herbicola* adsorbed to apple or cattail pollen were vectored to apple and pears flowers. Methods used to contaminate bees with inoculum are outlined. Quantitative observations on inoculum acquisition by bees and on deposition by bees on flowers are summarized. Factors that influence activity and foraging of bees are considered in relation to vectoring of inoculum and special methodology needed for studies of vectoring in the field. Suggestions regarding the potential of bee-vectored antagonists in suppressing flower infecting pathogens are presented.

Honey bees (*Apis mellifera*) were observed in recent studies to efficiently vector inoculum of microbial biocontrol agents to flowers of strawberry (Peng et al. 1992), raspberry (J.C. Sutton 1991, unpublished observations), apple and pear (Thompson et al. 1992). These observations were made a century after Waite (1891) reported for the first time that honey bees vectored a pathogen, *Erwinia amylovora*, to flowers of pear trees. For effective biocontrol of flower-infecting pathogens, it is likely that intensive vectoring of biocontrol agents is required. To achieve adequate vectoring of agents to flowers of crop plants, inoculum of the organisms must be suitably formulated to allow effective acquisition, transport, and deposition by bees.

Bees successfully vectored spores of *Gliocladium roseum*, *Epicoccum purpurascens*, *Alternaria alternata* and other fungal antagonists when formulated as powders with talc, pulverized corn meal, wheat flour, soya flour, or corn starch (Peng et al. 1992, Israel and Boland 1992). The bacterial antagonists *Pseudomonas fluorescens* and *Erwinia herbicola* were vectored to apple and pear flowers when adsorbed to pollen of apple or cattail (Thomson et al. 1992). The bees were contaminated with the formulations in special inoculum dispensers or pollen inserts inside hives. Bees acquired inoculum on their legs and bodies, and especially on their setae.

In a biocontrol study of fruit rot of strawberry caused by *Botrytis cinerea*, bees each acquired 88,000 - 1,800,000 (mean 570,000) cfu *G. roseum* in a talc formulation (5×10^8 cfu/g) and maintained an inoculum density of 1,600 - 27,000 cfu (mean - cfu) of the antagonist on each flower (Peng et al. 1992). Inoculum density in plots sprayed weekly with spore suspensions (10^7 conidia/mL) of *G. roseum* ranged from 300 to 15,000 cfu/flower. Propagule density was more stable and often higher on flowers of the bee-vectored treatment than in spray-treated flowers, but the treatments were about equally effective in suppressing incidence of the pathogen on stamens and petals, and in controlling fruit rot.

Efficiency of inoculum deposition on flowers by bees probably depends on subtleties in physical contact between the bee and the flower as well as the load and distribution of inoculum on the bee. Size and morphology of the flowers and

of the bees, and the activity and posturing of bees while on the flowers undoubtedly affect the amount of inoculum deposited and where it is deposited on the flower. In studies at the University of Guelph, bees delivered about 16 to 18 times more conidia of G. roseum per flower to strawberry than to raspberry. The formulation and concentration of inoculum used was the same in all studies. While strawberry flowers are much larger than raspberry flowers, and foraging frequencies by bees on the two types of flower may have differed, the bees also behaved differently on strawberry than on raspberry (J.C. Sutton, unpublished observations). In strawberry, bees tended to move actively over the face of the flower, often in a rotational pattern, and their legs and bodies frequently contacted the stamens and other flower parts. In raspberry however, the bees moved only slightly and tended to cling to the elongate stamens by means of distal portions of their legs, and achieved only minor body contact with the flower. While density of vectored inoculum on raspberry was low, the antagonist nonetheless effectively suppressed Botrytis fruit rot.

Many variables influence the frequency of visits by bees to flowers and may thus influence vectoring of biocontrol agents and the effectiveness of biocontrol. Cool temperature, wind, and rain generally discourage foraging by bees (Free 1968 a,b), however in our studies in strawberry, bees vectored high densities of G. roseum to the flowers under a wide range of weather conditions (Peng et al. 1992). Foraging in test plots or in commercial crops can be affected by the proximity and attractiveness to bees of other kinds of flowers in the area that compete as sources of nectar and pollen (Levin 1978). For example, biocontrol of B. cinerea in strawberry by means of bee-vectored G. roseum soon became ineffective when the bees preferentially visited freshly blooming rapeseed in nearby field plots (Peng et al. 1992). Chemical attractants can be used in some instances to maintain foraging in the target crop.

The mobility and foraging patterns of bees present special problems in field studies. Screens generally are needed to separate treatments with bees from those without bees, but may modify microclimate and exclude important pollinators. Bees confined in screen cages may forage and vector differently from freely-ranging bees. Screening of all treatments equalizes microclimatic modification but is impractical when plots or host plants are large, and can be costly. Vectoring of biocontrol agents will require special studies in commercial crops to determine the numbers, size and distribution of bee colonies needed for effective vectoring of microbial antagonists and for biocontrol. In bee-vectoring studies in Utah, the antagonist Pseudomonas fluorescens was detected on only 56% of apple flowers at 61 m from a hive, and on only 72% of pear flowers at 7 m from a hive, with an average population of 10^2 cfu per flower (Thomson et al. 1992). A strain of E. herbicola was detected on 92-96% of apple flowers in a 2.6 ha orchard (10-5700 cfu per flower). To encourage bees to establish foraging patterns in a crop as opposed to other plants in the area, it is important to introduce bee colonies shortly after the crop begins to flower.

Various bees potentially could be used to vector microbial antagonists to many kinds of plants for biocontrol of various flower-infecting pathogens. Several kinds of domesticated bees, including bumble bees (Bombus spp.), and leaf cutting bees (Megachile spp., Osmia spp.) as well as honey bees, may have potential as vectors. Wild species of halictid bees and andrenid bees also possibly could be used, and contaminated with biocontrol agents at bait stations. Various berry crops, orchard fruits, crucifer crops, beans, clovers, and cucurbits possibly could be protected by bee-vectored antagonists. Imaginative research could lead to effective, efficient, and environmentally safe biocontrol of many crop diseases by means of bee-vectored antagonists.

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