IOBC / WPRS

Working Group "Biological Control of Fungal and Bacterial Plant Pathogens"

and

EFPP

The European Foundation for Plant Pathology

Working Group on Biological Control

Biological Control of Sclerotium-forming Pathogens

Proceedings of a Workshop

Wellesbourne, Warwick (UK)
12-14 December 1994

Edited by
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IOBC wprs Bulletin
Bulletin OILB srop **Vol. 18 (3) 1995**

The IOBC/WPRS Bulletin is published by the International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palaearctic Regional Section (IOBC/WPRS)

Le Bulletin OILB/SROP est publié par l'Organisation Internationale de Lutte Biologique et Intégrée contre les Animaux et les Plantes Nuisibles, section Régionale Ouest Paléarctique (OILB/SROP)

Address General Secretariat:
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ISBN 92-9067-071-1

INTRODUCTION

This bulletin contains reviews and short research papers presented at the workshop "Biological control of sclerotium-forming pathogens". This workshop, held at Horticulture Research International, Wellesbourne, Warwickshire, UK, from 12-14 December 1994, was the third joint meeting of the Working Group on Biological Control of the European Foundation for Plant Pathology (EFPP) and the Working Group on Biological Control of Fungal and Bacterial Plant Pathogens of the International Organization for Biological Control, West Palaearctic Regional Section (IOBC/WPRS). The 3-day workshop with 26 contributions was attended by over 30 scientists from 10 European countries, including several East European ones.

The workshop was intended to bring together scientists with interests in biological control of a diverse range of pathogens all of which produce sclerotia. The first session focused on biological control of *Sclerotinia* species, the second session on biological control of other sclerotium-forming pathogens and the third featured overviews on the biological control of *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotinia* spp.

The workshop demonstrated that numerous possible approaches were available for successful biological control of sclerotium-forming pathogens. Understanding the life cycle of the pathogen, crop management practices and the ecology of the biological control agent, were clearly identified as key factors for achieving commercial success in applying specific antagonists to plants or soil. The need for improved selection, inoculum production and delivery systems for some antagonists to achieve cost effective control, were also highlighted. Significantly, solarization appears particularly useful for control of sclerotium-forming pathogens in areas with high insolation. Molecular biology in the area of transgenic plants was also shown to have potential as a biological control approach for the future.

Thanks are due to the following organisations for supporting the workshop financially or providing facilities free of charge:

— European Foundation for Plant Pathology (EFPP)
— Horticulture Research International (HRI)
— International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palaearctic Regional Section (IOBC/WPRS)

The IOBC/WPRS and the EFPP Working Groups are continuing their joint activities. Through the management committee consisting for the IOBC/WPRS of Claude Alabouvette (France), Yigal Elad (Israel) and Nyckle J. Fokkema (Convenor IOBC/WPRS, The Netherlands) and for the EFPP of Geneviève Défago (Switzerland), John Hockenhull (Convenor EFPP, Denmark) and John M. Whipps (UK), further joint workshops are planned. In addition, the committee is also involved in the planning stage of the next International Congress of Plant Pathology to be held in Edinburgh in 1998.

This bulletin contains the camera-ready text of the reviews and research contributions presented at the workshop. Many of the research papers are preliminary and primarily aimed to disseminate progress in biological disease control research at an early stage. Relatively minimal editing was carried out in most cases to allow rapid publication and so some minor inconsistencies remain. We thank all authors for their contributions.

The organisation of the workshop and the editing of the proceedings would not have been possible without the assistance of Sue Bewsey, Simon Budge, Tracey Greaves, Roger Williams and Chris Wood to whom the organisers are forever grateful. In addition, thanks must also go to all the other helpful and friendly HRI staff who made the workshop such a success.

The editors,

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Wellesbourne, February 1995

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BIOLOGICAL CONTROL OF *SCLEROTINIA* SPP.

EVALUATION OF SOLID-SUBSTRATE INOCULA OF *CONIOTHYRIUM MINITANS* AGAINST *SCLEROTINIA SCLEROTIORUM*

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Abstract

Soil incorporations of a range of solid-substrate inocula of *Coniothyrium minitans* were compared for antagonistic activity against *Sclerotinia sclerotiorum*, using a simple pot bioassay and a sequence of three glasshouse lettuce crops. In the pot bioassay, all ten inocula (barley, barley-rye-sunflower, bran-sand, bran-vermiculite, maizemeal-perlite, millet, oats, peat-bran, rice and wheat) evaluated almost completely inhibited carpogenic germination of sclerotia of *S. sclerotiorum*. Fewer sclerotia were consistently recovered after 20 wk from *C. minitans*-treated pots (9-29%) compared with the untreated controls (88%). High numbers (88-100%) of sclerotia recovered from pots treated with *C. minitans* were infected by the antagonist. The antagonist also spread to infect sclerotia in uninoculated control pots. Single soil-incorporations of five inocula (barley-rye-sunflower, maizemeal-perlite, peat-bran, rice and wheat) in a glasshouse trial controlled Sclerotinia disease, with only small differences between the types of inocula tested. At harvest of the second and third crops, *C. minitans* reduced sclerotial populations on the soil surface and over 70% of sclerotia recovered from *C. minitans*-treated plots were infected by the antagonist. *C. minitans* survived in soil in all solid-substrate inocula-treated plots for at least 39 wk at levels of 10^4-10^5 colony forming units cm^{-3} soil and spread to infect over 36% of sclerotia recovered from uninoculated control plots of both the 2nd and 3rd crop. The commercial implications of these results are discussed.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a pathogen of more than 360 plant species worldwide. In Europe, it regularly causes severe crop losses in beans, caraway, carrots, celery, lettuce, oilseed rape, potatoes and sunflowers. Losses are generally reduced by prophylactic spraying with fungicides. If the disease builds up in glasshouses, sclerotia of the pathogen can be eliminated by soil fumigation with chemicals such as methyl bromide or using steam sterilisation. However, the number of effective fungicides available is gradually decreasing, soil fumigation is becoming environmentally unacceptable and steam sterilisation is expensive. Consequently, alternative disease control methods, such as biological control, are urgently required. *Coniothyrium minitans* Campbell is a promising biological control agent of *S. sclerotiorum*, and has been shown to control the pathogen at low disease levels (McQuilken *et al.*, 1994 (and references therein)). Biological control with this antagonist has generally been obtained following soil incorporation of solid-substrate inocula prior to sowing or planting. However, a direct comparison of the biological control efficacy of these has not been made. This paper reports a simple pot bioassay and a sequence of glasshouse lettuce

trials conducted to compare a range of solid-substrate inocula of *C. minitans* for antagonistic activity against *S. sclerotiorum*.

Materials and Methods

Fungi

Sclerotinia sclerotiorum was isolated from diseased lettuce (Budge & Whipps, 1991). Sclerotia were produced on sterilised wheat grain, cv. Armada, following inoculation and incubation at 20°C for 3 wk (Mylchreest & Wheeler, 1987). Inocula of *C. minitans* (IMI 134523) were grown on ten solid-substrates (barley, barley-rye-sunflower, bran-sand, bran-vermiculite, maizemeal-perlite, millet, oats, peat-bran, rice and wheat) in spawn bags (22.5 x 56 cm) incubated at 18-20°C for 28 d (McQuilken & Whipps, 1995).

Glasshouse pot bioassay

Soil incorporations of different solid-substrate inocula of *C. minitans* were tested against carpogenic germination of sclerotia of *S. sclerotiorum*. Each solid-substrate was mixed thoroughly with soil (30% v/v) prior to filling ten replicate rectangular plastic pots (11 x 10 cm). Twenty sclerotia, enclosed in Terylene net bags (*ca* 5 x 5 cm, mesh < 2 mm), were buried in each replicate pot of soil-solid-substrate mix *ca* 1 cm beneath the surface. Pots were buried in soil in a glasshouse, with the soil surface in the pot at the same level as the surrounding glasshouse soil. Numbers of apothecia produced from sclerotia in each pot were counted at fortnightly intervals for 20 wk. After 20 wk, all sclerotia recovered were assessed for infection by *C. minitans* (Budge & Whipps, 1991).

Glasshouse lettuce trial

Soil was sheet-steamed and infested with sclerotia of *S. sclerotiorum* by planting a catch crop of lettuce and subsequently inoculating mature plants with the pathogen (Budge & Whipps, 1991). There were six treatments: (i) control, with no inoculum applied; (ii) barley-rye-sunflower; (iii) maizemeal-perlite; (iv) peat-bran; (v) rice; (vi) wheat. Inocula were incorporated into each plot (2.7 m^2) only once, one day before planting the first of three successive crops to give 0.6 litres inoculum m^{-2} for each treatment, a standard quantity used in previous experiments. Soil samples were taken from every plot at regular intervals and the survival of antagonists assessed by soil dilution plating (Whipps *et al.*, 1989). At harvest of each crop, assessments of disease, sclerotial numbers and infection by *C. minitans* were made for each plot (see Budge & Whipps (1991) for details).

Results

Glasshouse pot bioassay

In the glasshouse pot bioassay all soil incorporations of solid-substrate inocula of *C. minitans* significantly delayed and reduced the numbers of apothecia produced from sclerotia of *S. sclerotiorum* (Table 1). There were no differences between the different solid-

substrate soil incorporations of the antagonist. After 20 wk fewer sclerotia were consistently recovered from pots treated with the antagonist (9-29%) compared with the untreated controls (88%). High numbers (88-100%) of sclerotia recovered from pots treated with *C. minitans* were infected by the antagonist.

Glasshouse lettuce trial

There was no disease present in the first crop. In the second and third crops, all solid-substrate inocula treatments of *C. minitans* reduced the number of diseased plants when 44 and 82% of plants in control plots were diseased, respectively (Table 2). All solid-substrate inocula significantly reduced the number of sclerotia recovered at harvest in both the second and third crops. *C. minitans* infected sclerotia sampled from all plots, indicating spread of the antagonist to the controls. At harvest of either the second or third crops, levels of infection were always significantly higher in sclerotia recovered from plots treated with *C. minitans*. However, at either harvest there were no significant differences in infection of sclerotia recovered from plots treated with different solid-substrate inocula of the antagonist. Infection of sclerotia increased with time for both control and antagonist treatments. *C. minitans* exhibited a general decline in CFUs cm^{-3} soil with time in all plots treated with solid-substrate inocula (data not shown). However, the antagonist could still be detected within all treatment plots at levels of 2.2-15.5 x 10^4 CFUs cm^{-3} soil 39 wk after incorporation.

Table 1. Effect of different soil incorporations of solid-substrate inocula of *Coniothyrium minitans* on apothecial production, percentage recovery and infection of sclerotia of *Sclerotinia sclerotiorum* by the antagonist

Treatment	No. of apothecia after 20 wk Sum of fortnightly totals	% recovery	% infection by *C. minitans*
Control (nil)	105.2 ± 3.24[a]	88.0 ± 4.05	2.2 ± 2.22
Barley	3.2 ± 0.66	9.0 ± 3.31	100.0
Barley-rye-sunflower	5.8 ± 0.49	17.0 ± 2.99	100.0
Bran-sand	3.8 ± 0.80	14.0 ± 4.84	100.0
Bran-vermiculite	6.2 ± 1.90	18.0 ± 5.38	96.0 ± 3.99
Maizemeal-perlite	5.4 ± 1.69	29.0 ± 6.77	98.0 ± 2.00
Millet	4.6 ± 0.98	15.0 ± 4.18	100.0
Oats	4.6 ± 1.09	17.0 ± 3.38	88.0 ± 7.98
Peat-bran	3.4 ± 0.68	12.0 ± 2.99	100.0
Rice	5.0 ± 0.00	22.0 ± 4.89	90.0 ± 9.98
Wheat	5.0 ± 1.18	9.0 ± 3.31	100.0

[a] Values are means ± sample standard error of ten replicate treatment pots

Table 2. Effect of different solid-substrate inocula of *Coniothyrium minitans* on the percentage of *Sclerotinia sclerotiorum*-diseased plants (D), number of sclerotia recovered (NS) and infection (I) of sclerotia by the antagonist after successive lettuce crops.

Treatment	2nd Crop			3rd Crop		
	D	NS	I	D	NS	I
Control	44.4 (-0.28)[a]	47.8 (1.61)[b]	36.0 (-0.44)[a]	81.5 (1.51)[a]	107.8 (1.99)[b]	53.5 (-0.06)[a]
Barley-rye-sunflower	17.6 (-1.53)	11.8 (1.11)	74.5 (-1.50)	33.4 (-0.72)	22.2 (1.23)	83.8 (1.83)
Maizemeal-perlite	15.2 (-1.85)	20.4 (1.15)	74.8 (0.92)	41.4 (-0.31)	37.5 (1.60)	98.0 (3.54)
Peat-bran	14.3 (-2.02)	13.7 (1.02)	85.5 (2.10)	35.4 (-0.69)	35.8 (1.53)	91.2 (2.81)
Rice	16.1 (-1.95)	32.1 (1.19)	70.5 (1.29)	31.3 (-0.98)	35.0 (1.46)	91.5 (2.48)
Wheat	14.0 (-1.86)	23.0 (1.20)	89.0 (1.95)	25.0 (-0.95)	23.5 (1.42)	87.8 (2.37)
LSD	(0.568)	(0.434)	(0.951)	(0.312)	(0.238)	(1.089)

[a] Values in parentheses are angular transformations of percentages
[b] Values in parentheses are $\log_{10}$ (x) transformed

Discussion

There was little difference between the inocula in ability to infect sclerotia, reduce apothecial production or decrease Sclerotinia disease in lettuce. This suggests that any of these substrates could be suitable as basic materials for the development of commercial inocula of *C. minitans*. The only proviso must be that the application rate required is not excessive. On cost grounds, it has been suggested that industry would prefer to use inoculum produced by liquid fermentation which could then be formulated to provide a material with high viability, long shelf-life and which would be easy to apply. Work on this approach with *C. minitans* is currently underway.

Acknowledgements

This work was funded by the European Community and the Ministry of Agriculture, Fisheries and Food (MAFF) for England and Wales.

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USE OF *CONIOTHYRIUM MINITANS* IN INTEGRATED CONTROL OF *SCLEROTINIA SCLEROTIORUM* IN GLASSHOUSE LETTUCE

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Abstract

Pesticides used during the production of protected lettuce in the UK applied at up to twice the recommended dose and spray frequency rates had no effect on the ability of *Coniothyrium minitans* to survive and infect sclerotia of *Sclerotinia sclerotiorum* in soil. In a glasshouse trial, *C. minitans* applied to the soil before each of three lettuce crops reduced disease incidence from 12 to 7%; 32 to 19% and 84 to 44% in the first, second and third crops respectively, in comparison with the control. Disease incidence was further reduced to 1, 15 and 29% when a single application of iprodione per crop was combined with *C. minitans* soil application. The disease control achieved with the combined fungicide and *C. minitans* treatment was similar to that obtained with fortnightly sprays of iprodione.

Introduction

The fungal antagonist, *Coniothyrium minitans* Campbell is a well documented sclerotial mycoparasite of *Sclerotinia sclerotiorum* (Lib.) de Bary (Huang & Hoes, 1976; Turner & Tribe, 1976; Trutmann *et al.*, 1980; Whipps & Budge, 1990; Whipps & Gerlagh, 1992). *C. minitans* is also capable of reducing sclerotial development by the invasion of senescent plant tissue infected with *S. sclerotiorum* (Huang, 1977, 1983; Trutmann *et al.*, 1982; Whipps, 1987; Whipps & Gerlagh, 1992). At Horticulture Research International, the biological control potential of *C. minitans* has been investigated in a series of experiments and the mycoparasite was found to consistently reduce Sclerotinia disease in protected lettuce crops when disease levels were low, but as disease levels built up, control was lost (Lynch & Ebben, 1986; Whipps *et al.*, 1989; Budge & Whipps, 1991). Consequently, an integrated approach using *C. minitans* combined with reduced fungicide applications, may give a more efficient means of control than *C. minitans* alone. However, if this approach is to be used in commercial glasshouse lettuce production, then the pesticide compatibility of *C. minitans* needs to be investigated.

This paper describes the effect of a range of pesticides used during the production of lettuce in the UK, on the survival of *C. minitans* when applied as solid-substrate inoculum to soil in trays, and on its ability to infect sclerotia of *S. sclerotiorum*. In addition, a glasshouse trial evaluating the use of *C. minitans* in combination with reduced iprodione application to control *Sclerotinia* disease in three successive lettuce crops is outlined.

Materials and Methods

Fungi and inoculum production

The isolate of *S. sclerotiorum* was originally obtained from diseased glasshouse lettuce and was maintained on potato dextrose agar (PDA). Sclerotia used for the tray experiments were produced on sterilised wheat grain following inoculation with agar plugs containing mycelia of *S. sclerotiorum*. After incubation for three weeks at 20°C, sclerotia were harvested and those with a diameter of 3-4 mm were used immediately.

Coniothyrium minitans (IMI 134523) was originally isolated from a sclerotium of *S. sclerotiorum* (Turner & Tribe, 1976). Maizemeal-perlite inoculum of *C. minitans* used for the glasshouse trial was prepared by placing 2 l of flaked maizemeal and perlite (25:75% v/v) and 400 ml tap water into mushroom spawn bags (22.5 x 56 cm; Van Leer Ltd, UK). The bags were autoclaved, then inoculated with 100 ml of a spore suspension of *C. minitans* containing 2 x 10^6 spores ml^{-1}, obtained by flooding and scraping PDA Petri dish cultures. The bags were then incubated for two weeks at 18°C before use.

Tray experiments

Plastic trays (530 x 380 x 75 mm) with small holes in the bottom for drainage, were filled with a 2 cm layer of gravel, then a 5 cm layer of sieved soil (brickearth; silt-loam, Hamble series). Solid substrate inoculum (120 ml: 0.6 l m^{-2}) was added and lightly worked into the top 3 cm. Sclerotia (50) were lightly pressed into the soil surface. For each pesticide, there were four treatments: (i) sclerotia only; (ii) sclerotia and pesticide; (iii) sclerotia and *C. minitans* and (iv) sclerotia, *C. minitans* and pesticide. There were two trays per treatment and each pesticide set was repeated. Pesticides were applied at twice their recommended application rate and spray frequencies. The pesticides applied were: fungicides; dicloran, iprodione, propamocarb hydrochloride, thiram, fosetyl-aluminium, mancozeb, metalaxyl + thiram, toclofos-methyl, prochloraz, insecticides; pirimiphos-methyl, deltamethrin + heptenophos, dimethoate, malathion, pirimicarb, cypermethrin. All trays were incubated at 18°C for four weeks. Soil moisture was maintained by regular spraying with a hand sprayer. Soil samples (5 ml) were taken from three points within each tray immediately after addition of inoculum and each week thereafter, and survival of *C. minitans* was determined by soil dilution plating using PDA containing Triton X-100 (2 ml l^{-1}) and chlortetracycline (20 mg l^{-1}). After four weeks sclerotia were removed, surface-sterilised, bisected and placed on 15 mm diam. PDA plugs containing chlortetracycline. Numbers of viable and/or infected sclerotia were recorded after 14 days.

Glasshouse trial

A glasshouse chamber (13 x 9 m) was planted throughout with a crop of lettuce (cv. Hudson), which were inoculated with *S. sclerotiorum* to produce a natural population of sclerotia in the soil. At harvest the diseased debris was evenly spread throughout the chamber and the soil rotovated to a depth of 15 cm. Twenty plots (1.8 x 1.6 m) separated by 0.5 m paths were marked out. There were five treatments: (i) control, no treatment; (ii) single Rovral spray; (iii) fortnightly Rovral spray; (iv) *C. minitans* only and (v) *C. minitans* plus single Rovral spray. The treatments were randomized within the 20 plots to give four replicate plots

per treatment. Three crops were planted (72 plants per plot). Immediately before planting each crop, 1.7 l (0.6 l m^{-2}) of maizemeal-perlite inoculum was evenly applied to each appropriate plot and raked into the soil surface. Sprays of Rovral WP (50% iprodione; 0.5 g l^{-1} applied until run-off)) were applied at fortnightly intervals (treatment (iii)) or as a single spray per crop when disease symptoms proliferated (treatments (ii) and (v)). At harvest of each crop, the number of diseased lettuce were counted. The diseased material was left on the plots and from each plot, 60 sclerotia were sampled and their viability and/or infection by *C. minitans* assessed in the same way as the tray experiments.

Results and Discussion

Tray experiments

None of the pesticides applied affected either the survival of *C. minitans* in the soil or its ability to infect sclerotia (data not shown). Typically, the number of colony forming units (CFUs) declined very slightly from an initial level of 1.0-5.0 x 10^6 CFUs per cm^3 soil to 0.5-2.0 x 10^6 CFUs per cm^3 soil. Sclerotial infection by *C. minitans* was never below 80% and was usually 95-100%. With no apparent adverse effects upon *C. minitans* survival or its ability to infect sclerotia, these tests indicate that the maizemeal-perlite inoculum of *C. minitans* could be integrated into commercial lettuce production. However, full scale trials still need to be undertaken.

Glasshouse trial

Maizemeal-perlite inoculum of *C. minitans* alone, reduced disease incidence by nearly 50% in each crop in comparison to the control (Table 1). In combination with the single spray of Rovral, *C. minitans* reduced the number of infected plants still further, to a level very similar to that obtained using fortnightly sprays of Rovral. In contrast to previous experiments where only one application of *C. minitans* was used for a successive number of crops, the addition of inoculum before each crop in this trial, slowed down the disease build up with only 44% of plants diseased in the third crop. With the addition of a single spray of iprodione per crop (which had very little effect on its own) *C. minitans* was nearly as effective as regular sprays of Rovral.

Table 1. Effect of *C. minitans* soil incorporation and Rovral sprays on control of *S. sclerotiorum* in three lettuce crops

Treatment	Percentage number of diseased lettuce		
	1st Crop	2nd Crop	3rd Crop
1. Nil	12	32	84
2. Single Rovral spray	7	20	72
3. Fortnightly Rovral spray	5	5	21
4. *C. minitans*	7	19	44
5. *C. minitans* + single Rovral spray	1	15	29

Sclerotial viability was reduced by approximately 20% in both *C. minitans* treatments and sclerotia infection was between 90-98% (Table 2). As indicated by the tray experiments involving Rovral, the application of iprodione did not appear to effect the ability of *C. minitans* to infect sclerotia where the single spray was applied. In addition, *C. minitans* spread to infect equal numbers of sclerotia in both control plots and where Rovral was applied regularly.

Table 2. Effect of *C. minitans* soil incorporation and Rovral sprays on viability and infection of sclerotia of *S. sclerotiorum* at harvest of three lettuce crops

Treatment	% viability of sclerotia			% sclerotia infected by *C. minitans*		
	Crop			Crop		
	1st	2nd	3rd	1st	2nd	3rd
1. Nil	96.9	83.6	79.4	12.7	29.3	63.3
2. Single Rovral spray	92.9	90.5	86.1	16.9	35.7	73.9
3. Fortnightly Rovral spray	95.8	88.9	80.5	9.5	27.8	68.7
4. *C. minitans*	59.3	60.7	62.6	90.2	98.2	91.5
5. *C. minitans* + single Rovral spray	62.7	63.1	58.9	97.6	89.1	95.9

More glasshouse trials must be carried out to show consistent, statistically significant effects. Nevertheless, this trial indicates the feasibility of integrated control of *Sclerotinia* disease by combining *C. minitans* applications before each crop with fungicides applied only when necessitated by increased disease pressure.

Acknowledgements

This work was funded by the Biotechnology and Biological Sciences Research Council, the European Community and the Ministry of Agriculture, Fisheries and Food (MAFF) for England and Wales.

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BIOLOGICAL CONTROL OF WHITE MOULD (*SCLEROTINIA SCLEROTIORUM*) IN VARIOUS CROPS BY APPLICATION OF *CONIOTHYRIUM MINITANS*

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Abstract

Our general strategy of biological control of *Sclerotinia sclerotiorum* consists of treatment of a diseased crop or crop residue with a conidial suspension of *Coniothyrium minitans*. In a five-year field experiment with potato, bean, carrot and chicory in rotation, only bean was seriously affected by white mould in all years. In the plots treated with *C. minitans* the number of sclerotia, as scored by counting apothecia, was reduced by about 90% after each heavily diseased bean crop. This reduction was consistently found from the second year after the bean crop, as after the first year, the newly formed sclerotia were buried deeply through ploughing, which prevented their germination. When bean was again grown on the same plots four years after the initial bean crop, disease incidence in the plots treated with *C. minitans* was reduced by about 50%.

With a biennial carrot crop, a *C. minitans* conidial suspension was applied in autumn of the first year to prevent sclerotia formed on diseased plants from producing apothecia and thus inducing disease in the second year. Numbers of apothecia were reduced by more than 90%. This treatment, together with renewed *C. minitans* spray treatments of the crop in spring of the second year, resulted in a reduction of disease incidence of umbels from about 4% to 0.4%.

Introduction

Sclerotinia sclerotiorum causes white mould in a great diversity of crops. Only the Gramineae in the crop rotation are resistant. A diseased crop leaves numerous sclerotia at the soil surface, either exposed or within crop residues. Fungicide treatment of highly susceptible crops, such as bean and oilseed rape, has become standard practice to obtain good yield and quality, and to prevent soil contamination with sclerotia. The latter can severely limit the possibilities of using the soil for growing other susceptible crops for periods of over 5 years.

Coniothyrium minitans is well known as an effective mycoparasite of *S. sclerotiorum* (Whipps & Gerlagh, 1992). In principle it could be applied to reduce crop infection by ascospores of *S. sclerotiorum* and subsequent development of the pathogen in host tissues, but its application later in the vegetation period of the crop to infect and kill newly formed sclerotia seems even more promising in a long term crop management perspective.

We describe two field experiments, one over a five year period and the other in a biennial crop, which illustrate the possible use of *C. minitans* in preventing soil contamination with sclerotia, and its consequences for disease reduction.

Materials and Methods

A field experiment was started in 1990 on naturally contaminated soil. Four susceptible crops, potato, bean, carrot and chicory were grown in rotation on ten replicate plots (5 x 5 m^2) with *C. minitans*, *Trichoderma* spp and an untreated control as treatments. The treatments consisted of three crop sprays with a 5 x 10^6 cfu ml^{-1} conidial suspension of the antagonists at a rate of 1000 l ha^{-1} at about 15 day intervals in the second half of the vegetative period. In 1993 and 1994 the *Trichoderma* treatment was replaced by a single late season spray with *C. minitans*. To prevent spread of *C. minitans* to non-treated plots, all plots were situated in a 2 ha maize field. Numbers of surviving sclerotia were assessed by counting apothecia in 2 interrows per plot on several occasions when canopy closure and weather favoured sclerotial germination. The highest scores of the individual plots were considered to give the best impression of relative degree of contamination. Plants at both sides of 2 interrows were scored several times for disease incidence. Since disease incidence was only important in bean, the final, highest score of this crop only was used for analysis.

In a second experiment, carrots were sown at three row distances in a split-plot design on an artificially contaminated field. In autumn white mould disease appeared. On seven out of 13 plots (6 x 10 m^2), four sprays with a 5 x 10^6 cfu ml^{-1} *C. minitans* suspension were applied in September-October. In spring and early summer of the second year, after overwintering of the crop under plastic and straw, apothecia of *S. sclerotiorum* developed and were scored. Four more sprays with *C. minitans* were applied in June-July, and disease was scored in August, when the crop was ripe, by assessing the percentage of diseased umbels.

Results and Discussion

In 1990 and 1991 no effect of the treatments on numbers of apothecia and disease incidence could be established. But assessment of infection of sclerotia by *C. minitans* had shown about 90% destruction of sclerotia by the mycoparasite in the *C. minitans* treated plots (Gerlagh *et al.*, 1993). The apothecial counts of 1990 merely reflect the natural contamination of the soil. Due to different time and degree of maximum soil coverage by the crop canopy of the four crops, counts differed per crop (Table 1). This was also observed in the other years. Generally potato seemed to yield the highest number of apothecia. In 1991, all counts at the usual period after crop canopy closure were low, except for potato. This was presumably caused by extremely wet weather in June, which caused germination of sclerotia on practically bare soils. They were not counted then to prevent damage to the soil structure of the field. Only potato had a good soil coverage at that time, and apothecia were counted soon after the frequent rains stopped. The very low number of apothecia resulted in the low number of diseased bean plants (Table 2). In all other years bean was highly infected, whereas the other crops showed hardly any disease.

Table 1. Effect of crop treatments with antagonists on counts of apothecia of *S. sclerotiorum* under various crops in rotation

Crop	Treatment	1990	1991	1992	1993	1994
Potato	*C. minitans*	60[1)]	30	11	**26**	0
	Trichoderma spp.	57	42	16	**148**	0
	Control	91	48	11	**95**	0
Bean	*C. minitans*	**17**	5	2	5	**4**
	Trichoderma spp.	**24**	3	11	11	**36**
	Control	**5**	5	6	8	**14**
Carrot	*C. minitans*	36	**3**	9	11	5
	Trichoderma spp.	36	**3**	10	16	4
	Control	42	**3**	6	23	2
Chicory	*C. minitans*	21	14	**11**	14	2
	Trichoderma spp.	23	14	**122**	25	47
	Control	40	13	**100**	25	29

1) Each figure represents the sum of the highest score per plot of several counts at different times for 10 m row length and 10 plots.

N.B. The *Trichoderma* spp. treatment was replaced in 1993 and 1994 by a single spray with *C. minitans*.

Table 2. Effect of spraying bean crops with *Coniothyrium minitans* and *Trichoderma* spp. on the number of apothecia and disease incidence

Factor	Treatment	1990	1991	1992	1993	1994
Apothecia[1)]	*C. minitans*	17	5	2	5	4
	Trichoderma spp.	24	3	11	11	36
	Control	5	5	6	8	14
Disease incidence[2)]	*C. minitans*	518	55	428	218	529
	Trichoderma spp.	501	42	393	243	1023
	Control	343	46	242	284	964

1) Sum of the highest score of counts at different times for 10 m row length of 10 plots;

2) Sum of the number of infected plants at both sides of a 10 m interline of 10 plots, each scored at the time of maximum disease incidence.

N.B. The treatment with *Trichoderma* spp. was replaced in 1993 and 1994 by a single spray with *C. minitans*.

In 1992, the first effects of *C. minitans* treatment were visible in the scores of apothecia in chicory (Table 1). This crop grew on the plots where bean was cropped in 1990. Due to ploughing in autumn, sclerotia produced on the 1990 bean crop, were buried deeply. Only the ploughing of autumn 1991 brought them to the surface again. Thus the number of apothecia in 1992 reflects the effect of the *C. minitans* treatment of two years before. In the potato crop, produced on ridges, which further mixes the soil, the situation in 1993 confirmed the findings of 1992. The bean crop of 1994 showed the effect once more. The disease incidence of beans in 1994 (Table 2) reflects the effect of the reduction in number of apothecia. The *C. minitans* treatment resulted in roughly halving the number of diseased plants compared to the other treatments.

In 1993 the effect of spraying the bean crop with *C. minitans* in 1991 was not traceable in chicory. This could be explained by the low disease incidence in 1991, which led to a low soil contamination. In 1994, the effect in chicory was again as expected. No effect of the *Trichoderma* treatment was ever found. The replacement of this treatment by a single spray with *C. minitans* was not expected to have any visible effect before 1995.

Spraying of a biennial carrot crop in autumn of the first year strongly reduced the number of apothecia in spring of the second year. The wider row distance resulted in a lower number of apothecia per m^2, noticeable only in the control treatment (Table 3). Since no ploughing is done between first and second year, old and superficial sclerotia will mostly get exhausted in the first cropping year. Apothecia in the second year depend on sclerotia formed in autumn of the first year on the infected crop. The treatment with *C. minitans* resulted in virtual absence of inoculum in spring and summer of the second year, and this, together with more antagonist sprays, resulted in a reduction of disease incidence of umbels from about 4% to 0.4% (Table 3).

Conclusion

C. minitans is a promising antagonist for biocontrol of *S. sclerotiorum* (Whipps & Gerlagh, 1992). It has successfully been applied to soil in glasshouse (Whipps & Budge, 1992) and field conditions (Şesan & Csép, 1992). Reduction in disease incidence due only to spraying a crop with *C. minitans* has not been described, but disease severity and production of sclerotia have been shown to decrease (Trutmann *et al.*, 1982a). Consequently *C. minitans* holds promise for biocontrol by decreasing the level of soil infestation as already implied by Trutmann *et al.* (1982b). In our experiments, this strategy proved to be correct. Sclerotial number in soils decreased by at least 90% due to application of *C. minitans* on an infected crop, and crops grown under the reduced inoculum pressure showed significant reduction in disease incidence compared to the control.

Table 3. Effect of spraying of *C. minitans* in the first and second year of a biennial carrot crop, sown at varying row width, on the number of apothecia and disease incidence in the second year

Treatment	Row width (cm)	Apothecia[1)]	Disease incidence[2)]
Control	20	3.07 *a*	3.34 *a*
	40	1.35 *b*	4.79 *a*
	60	0.60 *b*	3.67 *a*
C. minitans	20	0.11 *b*	0.73 *b*
	40	0.04 *b*	0.26 *b*
	60	0.13 *b*	0.39 *b*

1) Number per m^2
2) % of umbels infected

Numbers in the same column followed by different letters are significantly different ($P \leq 0.05$)

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BIOLOGICAL CONTROL OF *SCLEROTINIA SCLEROTIORUM* IN OILSEED RAPE AND SUNFLOWER WITH THE FUNGAL ANTAGONIST *CONIOTHYRIUM MINITANS* AND IMPROVEMENT OF ITS USE

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Abstract

Field trials were carried out to investigate the effectiveness of applications of *Coniothyrium minitans* against *Sclerotinia sclerotiorum* on oilseed rape and sunflower. In spite of a low infection pressure in the year of the investigations, a significant reduction of the percentage of infected oilseed rape plants was obtained when *C. minitans* was applied to the upper soil layer, in one case. However, the maximal disease reduction caused by *C. minitans* was only 30%, which is not good enough for practical use. Coating sunflower seed with 5×10^7 spores of *C. minitans* reduced Sclerotinia root infection of sunflower plants effectively from 46 to 11%, while 5×10^5 spores per grain failed to protect the plants against infection satisfactorily, with the infection of sunflowers reduced to only 25%. To improve *C. minitans* as a biological agent some selection work was carried out. Strains with constant, well-distinguishable characteristics could be obtained by the selection of single pycnidia. These strains, which are believed to be pure genotypes, showed considerable differences in their antagonistic effectiveness. Further selection was used to obtain stable genotypes suitable for mass production.

Introduction

In Germany, two field crops, oilseed rape and sunflower, are particularly susceptible to infection by *S. sclerotiorum*. In the northern part of Germany, yield losses in oilseed rape up to 50% have been observed (Hornig, 1983). In sunflower, no successful chemical products against the disease are known and, in oilseed rape, the application of pesticides is associated with difficulties. Essentially, when the decision to apply pesticides has to be made, it is uncertain whether the disease will spread. Therefore, farmers spend a lot of money on prophylactic fungicide treatments unnecessarily in many years. However, if a biological control agent active against the sclerotia of the pathogen in soil was available, a longer-lasting effect could possibly be achieved even if the disease subsequently failed to spread. Another disadvantage of the use of chemical pesticides is the timing of application. Application has to be carried out during flowering and, therefore, some mechanical damage to the plants occurs. Driving on the field during flowering causes yield losses of 2.5 to 5% (Gröner, 1984). By the use of a biological preparation, such losses would be avoided because its application could take place before sowing. A further advantage of using a biological pesticide is that environmental pollution resulting from the application of chemical products could also be reduced.

The antagonistic fungus *C. minitans* is considered to have potential as a biological pesticide for use against *S. sclerotiorum* (Whipps & Gerlagh, 1992). However, there are still

a lot of problems which have to be solved before a commercial use of *C. minitans* is possible. The objective of the present work was to increase the knowledge of suitable application conditions, as well as improve the selection of strains of *C. minitans* with both high levels of spore production and effectiveness as antagonists against *S. sclerotiorum*.

Origin of *Coniothyrium minitans* isolates

Isolates of *C. minitans* were recovered from different soils in Mecklenburg-Vorpommern in northern Germany by baiting with sclerotia of *S. sclerotiorum* for 2 months from September to October, 1990. Because host plants of *S. sclerotiorum* had been grown there several times before, all used soils were naturally infested with *C. minitans*. Twenty three isolates of *C. minitans* were obtained.

Field trials with spore suspensions applied to soil

The trials were carried out at two different locations in August 1993. The first location was Johannisdorf in Schleswig-Holstein, which had a heavy clay soil and the other was Biestow in Mecklenburg-Vorpommern, which had a more sandy soil. Both trials were carried out in the same manner with the only difference being that in Johannisdorf three different spore concentrations were used, in contrast to only two in Biestow.

After infestation of the trial plots with 60 sclerotia per m^2 and preparation of the soil for sowing, three different isolates of antagonists were applied by spraying a spore suspension onto the soil surface. The amount of the suspension was 50 ml/m^2 and the concentration was 1×10^5, 1×10^6 and 1×10^7 spores per ml respectively. A spore suspension not only provides a very regular distribution of fungus propagules, but also has the advantage that the spores enter the soil layer where sclerotia are located which are responsible for the infection of oilseed rape in the following spring. Spores are covered with soil either during the last working cycle before sowing or at sowing and, consequently, there is enough moisture to protect them against drying and to induce their germination.

The trials were evaluated in July 1994 by counting the numbers of infected and uninfected plants and the infection percentage was determined. The result of the trial in Johannisdorf is shown in Fig. 1.

Only the highest level of *C. minitans* resulted in a reduction of infection; between 15 and 20% in comparison with the untreated control. No differences between the isolates could be observed. In contrast, in Biestow (Fig. 2), the *C. minitans* isolates consistently reduced Sclerotinia infection of oilseed rape by 25-30% compared with the untreated control. The most effective isolate was P VIII 3 followed by P VIII 2 and P VIII 6. Unfortunately, the differences between the isolates were not statistically significant. Here, even the lower spore concentration had a reducing effect on the infection rate. Presumably the conditions for control by *C. minitans* were better in Biestow than in Johannisdorf. This effect could probably be a result of the lighter and warmer soil.

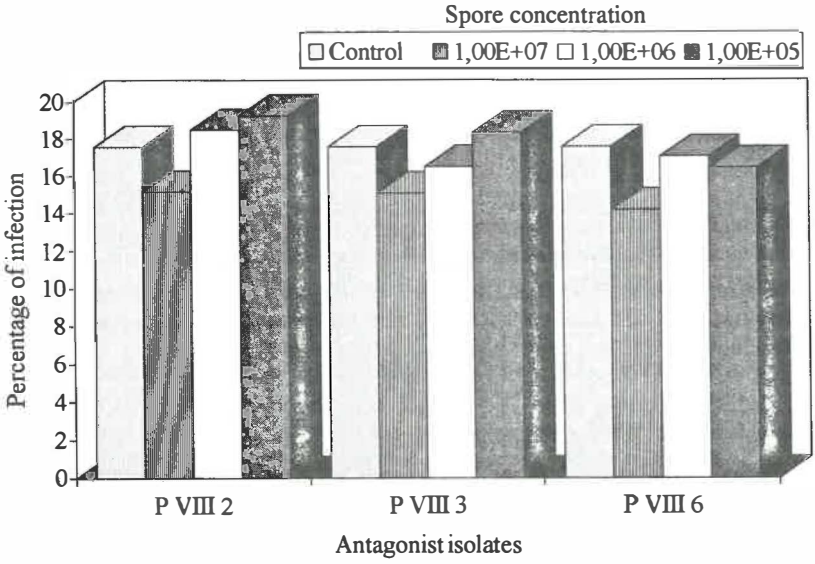

Fig. 1. Effectiveness of three isolates of *Coniothyrium minitans* at three different spore concentrations on the percentage infection of oilseed rape by *Sclerotinia* in Johannisdorf

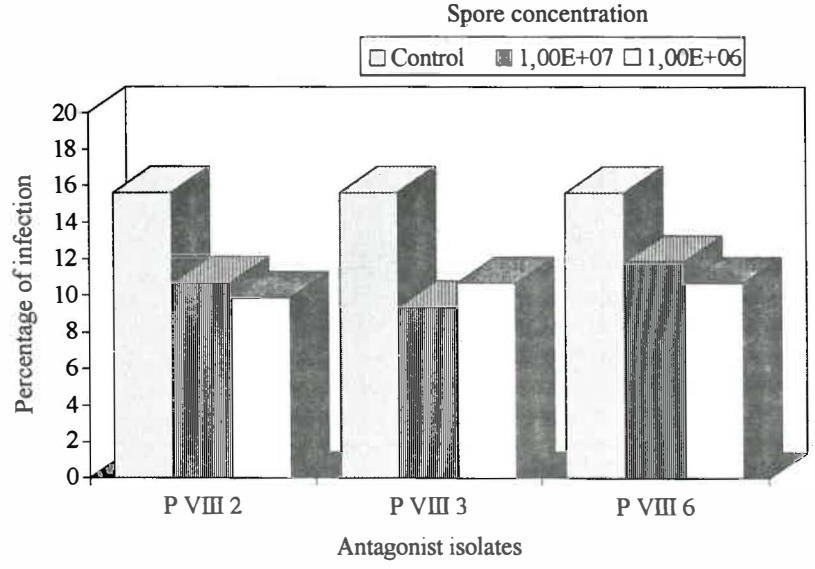

Fig. 2. Effectiveness of three isolates of *Coniothyrium minitans* at two different spore concentrations on the percentage infection of oilseed rape by *Sclerotinia* in Biestow

Despite *C. minitans* reducing Sclerotinia disease consistently, the degree of reduction was insufficient to recommend any of the treatments for practical use. There are several possible reasons for this. For example, the weather conditions in 1994 led to a only very limited infection pressure during flowering. Therefore, in spite of the additional soil infestation with sclerotia, the infection was not as great as necessary for a serious disease development in the plots. Also, because ascospores are dispersed by wind, it is likely that the *Sclerotinia* infection in one plot could have originated from the apothecia of another plot, leading to a more even level of infection than expected. Further, the isolates of *C. minitans* used for the trials had not been selected for their antagonistic effectiveness before application and may not have been very active.

Field trials with spores applied as a seed coating treatment

The seed treatment trial was carried out with the help of Ernst Benary Seed Growers Ltd. in Austria using plots (30 m^2) in which approximately 800 sunflower plants were sown at the end of May 1994. Previously, the trial area had been infested with sclerotia of *S. sclerotiorum* using 240 sclerotia/m^2. The seed used for the trial had been coated with a ground wheat bran culture of *C. minitans* using Gum Arabic as a sticker. Isolate P VIII 3 at a rate of 5 x 10^5 and 5 x 10^7 spores per grain was used with two replications.

Because of a very hot and dry summer, infection of the plants was not observed until August. The evaluation of the trial was carried out at the end of September by counting the plants with infected roots and relating them to the total number of plants per plot (Fig. 3). With 5 x 10^7 spores per grain, a significant reduction of *Sclerotinia* infection from 46% in the control to 18% was found. Therefore, it seems to be possible to inhibit the root infection rate of sunflower plants by an application of *C. minitans* to the seed. The same conclusion resulted from trials performed in Romania (Şesan, 1994).

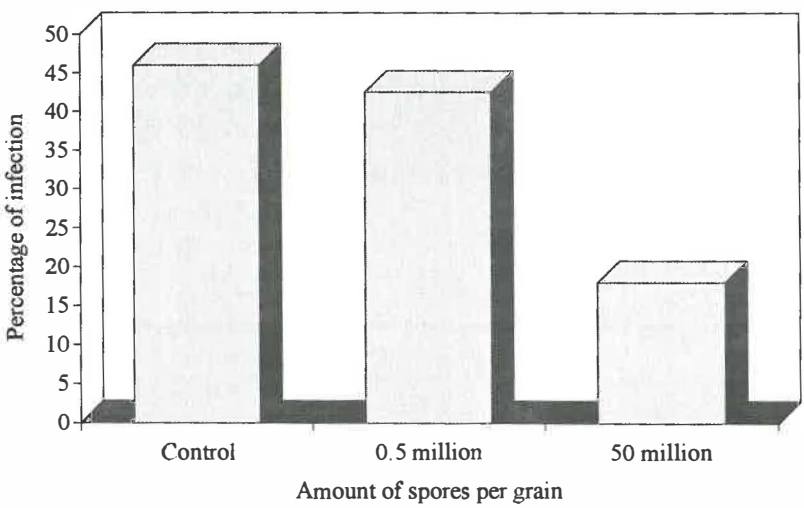

Fig. 3. Effect of application rate of *Coniothyrium minitans* on sunflower seed on percentage infection by *Sclerotinia sclerotiorum* in the field

The method of application of *C. minitans* by coating the seed can only be used in cases where plants are infected via the root or the stem bases. In those cases where the upper parts of plants are infected by the ascospores, an application of *C. minitans* to the seed would not be effective.

Selection of strains of *C. minitans* with high levels of spore production and antagonistic ability

All investigations described were carried out with *Coniothyrium* isolates which were not specifically selected for use in a formulated pesticide. For this purpose, isolates must have two main characteristics. Firstly, they have to be suitable for mass production and secondly they have to be effective antagonists.

Because a suspension of spores is the most suitable formulation for an even distribution of a large amount of propagules in the soil it was decided to use these propagules throughout. However, there are some disadvantages. For example, the bare spores could be very sensitive to unfavourable environmental conditions. Nevertheless, one of the most important purposes of our efforts in the development of a biological plant protection preparation was the selection of isolates with a high spore production ability. To obtain such isolates, the following selection scheme was developed.

From all 23 original isolates a spore suspension was produced and spread onto a malt yeast agar containing streptomycin sulphate. Immediately after application, the Petri dishes were incubated in two different ways. They were either incubated under UV light (254 nm wavelength) for 8 hours as a mutagenic treatment before being placed at 20°C in the dark, or they were immediately incubated in the dark without any exposure to UV. After 10 days, the number of pycnidia produced were used to distinguish one isolate from another isolate. On these differences, which did not depend on the exposure to UV, new strains were selected by removing single pycnidia from areas with especially good pycnidial development. These pycnidia were placed onto a fresh nutrient agar and those cultures with the best sporulation were used for another cycle of selection. After the second selection cycle, 32 new *Coniothyrium* strains with an especially good sporulation ability had been obtained (Fig. 4). On average the selected strains yielded 1×10^9 spores per Petri dish while the original isolates yielded approx. 2×10^8 spores per Petri dish.

The best strain, designated as CON/M 90-23, produced 2.8×10^9 spores per Petri dish. The culture of CON/M 90-23 in a swing solid-state reactor resulted in a yield of 2×10^{12} spores per kg culture substrate. This amount would probably be sufficient for the treatment of 1 hectare farmland.

Another result of the selection was that all strains represented single genotypes with characteristic growth patterns in culture. The use of only one genotype with identifiable characteristics could be very important for protection by patent. Apart from this, the use of only one genotype or a mixture of a few well-characterised genotypes helps to guarantee that all supplies of the preparation have identical characteristics.

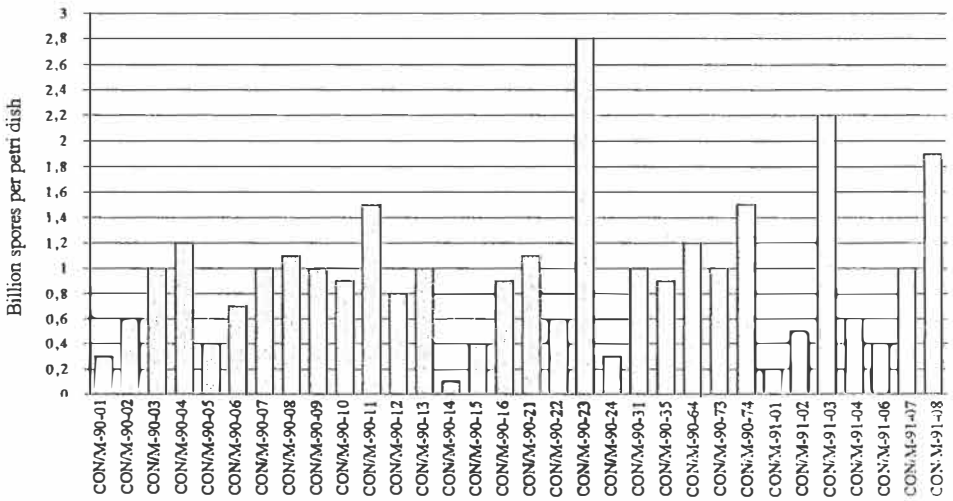

Fig. 4. Spore production ability of 32 selected strains of *Coniothyrium minitans* (values are means of 4 replications)

Subsequently, the selected genotypes were used to investigate their antagonistic ability against sclerotia of *S. sclerotiorum* in tests using silver sand under controlled environmental conditions. The selection of effective genotypes at known temperatures is very important to assure effectiveness under natural conditions in the field. For the test, Petri dishes were filled with 120 g of silver sand and the sand was moistened with 28 ml tap water together with 0.318 ml of a *Coniothyrium* spore suspension containing 1 x 10^6 spores per ml. The amount of spore suspension corresponded to an application rate in the field of 500 l/hectare. After the addition of the spore suspension, 25 sclerotia were placed into the sand in each Petri dish. Petri dishes were incubated in the dark at a temperature of 15°C for the first 4 weeks and 12°C for the remaining 8 weeks. After a 12 week incubation sclerotia were cut into slices and the nature of the sclerotia tissue was examined. On the basis of this examination a damage index from 0 (no damage) to 100 (most damage) was calculated (Fig. 5).

All *Coniothyrium* strains were able to damage the sclerotia of *S. sclerotiorum* in comparison to the untreated control, even though the control sclerotia were also damaged. This reflected infections by other micro-organisms, particularly members of the Mucorales. The best strain, designated as CON/M/91-08, caused a damage index of more then 60%. By using this strain, more than 90% of the sclerotia were damaged, most of them heavily.

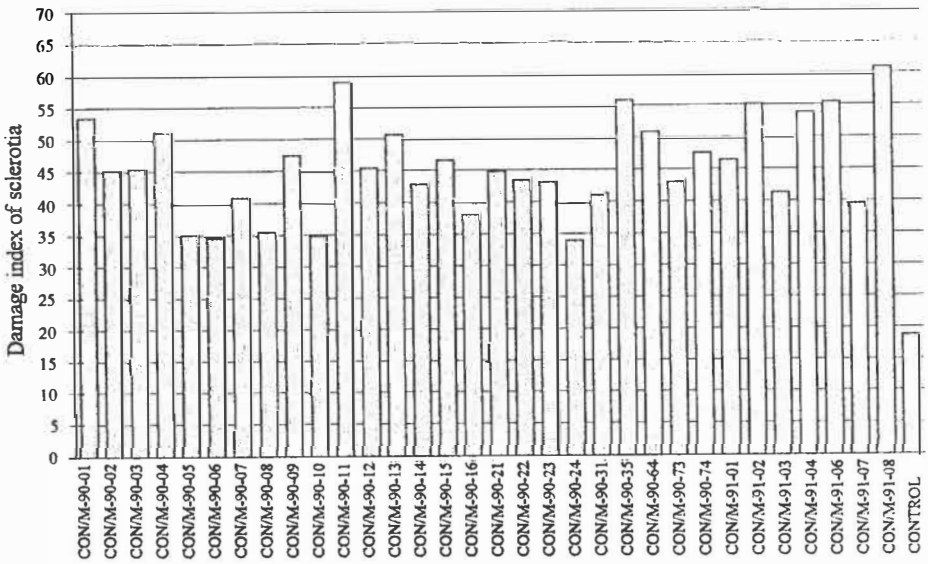

Fig. 5. Ability of 32 *Coniothyrium* strains to damage sclerotia of *S. sclerotiorum* after incubation at 15°C for 4 weeks followed by 12°C for 8 weeks (values are means of 4 replications)

The results show that there are considerable differences concerning the antagonistic activity between different genotypes. The difference between the least active strains (CON/M/90-05, CON/M/90-06, CON/M/90-08, CON/M/90-10 and CON/M/90-24) and the most active strains (CON/M/90-11, CON/M/90-35 and CON/M/91-08) was significant at P = 0.05.

As the result of the selection experiments, one *Coniothyrium* strain (CON/M/91-08) with both a high level of spore production and antagonistic ability was obtained.

Conclusions

The addition of *C. minitans* into the upper soil layer is suitable to reduce the infection rate of oilseed rape. However, its activity as an antagonist depends on the amount of fungal propagules applied, on the effectiveness of the isolate used and on many edaphic factors including temperature. Future field trials to investigate the effectiveness of *C. minitans* against *S. sclerotiorum* in oilseed rape have to be carried out avoiding the spread of ascospores from one plot to another. Use of large untreated strips between treated plots may help here.

Coating sunflower seed with propagules of *C. minitans* can be effective against Sclerotinia root infection of sunflowers. An application rate of 5×10^7 spores per grain, reduced the infection of sunflowers in a field trial from 46 to 18%, but a lower rate (5×10^5 spores/grain) was not effective. Strains with constant well distinguishable characteristics could be obtained by the selection of single pycnidia. These strains, which are thought to be pure genotypes, showed considerable differences in their antagonistic effectiveness. Therefore, for further work in regard to the development of a registered plant protection preparation, only selected highly efficient genotypes, used singly or in mixtures, should be considered.

In conclusion, it can be said that there are good chances for the development of a biological pesticide against *S. sclerotiorum* using the fungal antagonist *C. minitans*. However to reach this goal problems such as the high production cost (Whipps & Gerlagh, 1992), will have to be solved.

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INVESTIGATIONS ON *CONIOTHYRIUM MINITANS* AND *TRICHODERMA* SPP. TO CONTROL DISEASES OF INDUSTRIAL CROPS CAUSED BY *SCLEROTINIA SCLEROTIORUM*

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Abstract

The paper presents the main results obtained in field trials during 1988-1994 where the antagonistic and hyperparasitic fungi *Coniothyrium minitans* and *Trichoderma* spp. (*Trichoderma viride* and *T. harzianum*) were tested for ability to control white rot (*Sclerotinia sclerotiorum*) in sunflower and soybean. The efficacy of soil treatments with *C. minitans* was similar to that of seed treatment with dry biomass of *T. viride* (Td_{50}) and the commercial biofungicide Trichodex 25 WP (*T. harzianum* - T-39), and comparable with treatments with standard specific chemical fungicides, such as Sumisclex 50 WP or Metoben 70 PP. The biological treatments also have the advantage of protecting the agroecosystem of both crops.

Introduction

White rot caused by the fungus *Sclerotinia sclerotiorum* is a major disease of crops throughout the world. Although there are numerous chemicals available for control of white rot, more reports of biological control agents for use against sclerotial pathogens are appearing in the literature.

Early investigations in Romania on biological methods to prevent white rot in sunflower and soybean started at RIPP-Bucharest, concentrating mainly on the use of *Trichoderma* spp. (Şesan, 1985, 1986, 1993), and *Coniothyrium minitans* Campbell (Şesan & Crişan, 1988; Şesan, 1989; Şesan & Csép, 1991, 1992, 1993, 1994). These investigations attempted to demonstrate the effectiveness of this approach through experimental means, and highlight the practical opportunities for preventing *Sclerotinia*-type diseases in sunflower and soybean, crops of economic importance for this country.

Materials and Methods

Randomized field trials have been performed during 1988-1994 at the Agricultural Experimental Station Oradea (AERS), District of Bihor, using sunflower cvs and hybrids, and soybean cvs provided by the Research Institute for Cereals and Industrial Plants, Fundulea.

The experimental variables consisted of treatments with *Coniothyrium minitans* (C.m.) cultured on potato dextrose agar applied at a rate of 250 g/m^2 to soil in the planting pits at sowing (April) or seed treatments with the biofungicide Trichodex 25 WP

(*Trichoderma harzianum* Rifai - T-39), manufactured by Maktheshim Agan Company (Israel), and dry *Trichoderma viride* Pers. ex S.F.Gray biomass (isolate Td_{50}), produced at the RIPP-Bucharest, both applied at rates of 2 g/kg seed. Each variable was replicated three times in each trial. The soil was artificially inoculated with 7-10 day-old cultures of *S. sclerotiorum* grown on autoclaved oat seeds. Efficacy of treatments with *C. minitans* and *Trichoderma* spp. was compared to that of the chemical fungicides Sumisclex 50 WP, Metoben 70 PP, at 2 g/kg seed; AC-8, 3 g/kg; Tiradin 75 PP at 4 g/kg, used as standards, as well as to an untreated control.

Frequency (F%) of diseased plants and yield (kg/ha) were recorded and data were analysed using Abbott's formula. Frequency of healthy and diseased plants, and seed yield were also examined using ANOVA.

Results and Discussion

Disease development

In this country, *S. sclerotiorum* attack involved mycelium overwintering within seeds, or appeared either as a result of myceliogenic sclerotia germination in soil (collar rot and seedling dieback), or due to ascospore infection on flowering primordia, on stems (stalk rot, white rot), petioles, leaves and inflorescences (head rot) (Figure 1).

Sunflower trials

In sunflower (Table 1), the environmental conditions in 1988, 1989 and 1992 were less favourable for white rot development, and disease frequency in control plots ranged between 12.5% and 19%. In the other years (1990, 1991, 1993 and 1994) climate conditions were adequate for disease development with the frequency of attack in controls between 28.5 to 35.5%.

In all biological treatments some differences in efficacy have been observed, presumably due to varying meteorological conditions and also the cv/hybrid used.

Coniothyrium minitans applications to soil provided the highest efficacy (E% = 60.0-73.8) during 1993-1994 and 1988-1989, as compared to the chemical standard Metoben/Sumisclex 50 WP (E% = 42.6-89.9), with efficacy being correlated to the low white rot attack frequency (F% = 5.0-8.5) during this period. In 1990-1992 effectiveness of *C. minitans* treatments was lower, ranging between 43.0 and 48.0%, than the chemical standard (E% = 47.5-76.0).

Efficacy of seed dressing with *T. viride* (Td_{50}) dry biomass reached high values, between 62.0 and 76.3% in 1993-1994 and 1988-1989, whilst its effect was more reduced in 1990-1992 (E% = 4.7-48.0). Seed treatment with Trichodex 25 WP, applied only in 1991-1994, proved to be efficient (E% = 43.7-78.5) as a biological product.

Fig. 1

BIOLOGICAL CYCLE OF SUNFLOWER SCLEROTINIA – DISEASE (after CSÉP, 1993)

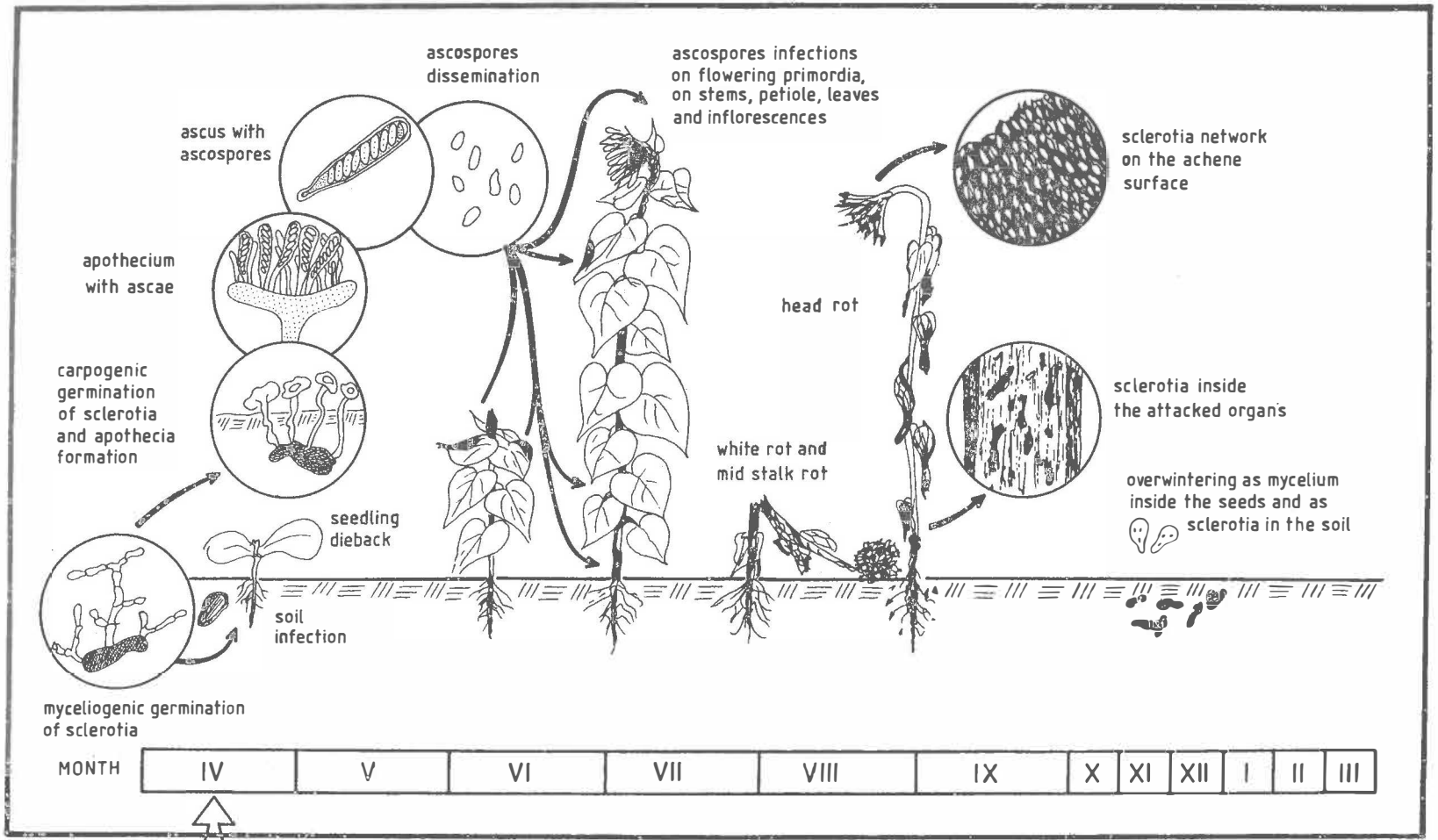

Table 1. Efficacy of treatments with *Coniothyrium minitans* (C.m.) and *Trichoderma* spp. (Td_{50}, Trichodex 25WP) to prevent sunflower white rot (*Sclerotinia sclerotiorum*) at Oradea (1988-1994)

Year/Cv./Hybrid	Treatment	F %	E %	CE %	Seed yield kg/ha
1988	C.m.	7.0	63.0	148.0	-
Florom	Td_{50}	7.3	62.0	146.0	-
305	Metoben	10.9	42.6	100.0	-
	Control	19.0	-	-	-
1989	C.m.	5.0	60.0	79.0	-
Record	Td_{50}	4.3	65.6	86.0	-
	Metoben	3.0	76.6	100.0	-
	Control	12.5	-	-	-
1990	C.m.	20.0	43.7	57.5	1580
Fundulea	Td_{50}	19.0	46.5	61.2	1500
90	Metoben	8.5	76.0	100.0	1750
	Control	35.5	-	-	1450
1991	C.m.	19.5	43.0	90.5	1375*
Florom	Td_{50}	20.0	41.7	87.8	1410**
328	Trichodex 25WP	19.3	43.7	92.0	1450**
	Sumisclex	18.0	47.5	100.0	1500**
	Control	34.3	-	-	1050
1992	C.m.	6.5	48.0	80.0	1450
Florom	Td_{50}	6.5	48.0	80.0	1500
328	Trichodex 25WP	6.0	52.0	87.0	1530*
	Sumisclex	5.0	60.0	100.0	1550*
	Control	12.5	-	-	1250
1993	C.m.	8.5	73.8	82.1	1490
Florom	Td_{50}	7.7	76.3	84.9	1550
328	Trichodex 25WP	7.0	78.5	87.3	1580*
	Sumisclex	3.3	89.9	100.0	1590*
	Control	32.5	-	-	1300
1994	C.m.	7.9	72.3	80.8	1400
Decor	Td_{50}	7.0	75.4	84.2	1425
	Trichodex 25WP	6.8	76.1	85.0	1440
	Sumisclex	3.0	89.5	100.0	1525**
	Control	28.5	-	-	1250

*, ** Significantly different from the control at $P < 0.05$ and 0.01 level respectively
F % - Disease frequency; E % - Efficacy of control;
CE % - Control effect in comparison with standard

In terms of sunflower seed yield (Table 1), in 1990-1994 no marked differences between soil treatments with *C. minitans* (1,375-1,580 kg/ha), and Td_{50} seed treatment (1,410-1,550 kg/ha) occurred; all values were slightly lower than the standard (1,500-1,750 kg/ha) but always higher than the untreated control (1,050-1,450 kg/ha). The highest seed yield gains have been obtained with the chemical standard (275-450 kg/ha), followed by Trichodex 25 WP (190-400 kg/ha), *C. minitans* (130-325 kg/ha), and Td_{50} (50-360 kg/ha).

Our data obtained with *C. minitans* in sunflower are in agreement with those obtained by various workers in different areas of the world (see Whipps & Gerlagh, 1992). Our contributions to the study of biological control in the development of IPM technology for the sunflower crop using *Trichoderma* spp., particularly *T. viride*, extend our previous work (Şesan, 1985, 1986, 1989, 1993), and bring additional reasons to include such non-polluting elements in the protection technology of this crop. Trichodex 25 WP, recommended by its producer (Maktheshim Agan - Israel) for season-long control of grey rot (*Botrytis cinerea*) in grapevine, was also trialled by us as a sunflower seed dressing, with some success. This is significant as the crop is equally attacked by *B. cinerea* and by *S. sclerotiorum* and a single biocontrol agent may control both diseases.

Soybean trials

In soybean (Table 2) white rot frequency in the controls was similar throughout the 7 year experimental period, varying between 8.5 and 20.5%; these values were lower than those in the sunflower controls. All biological treatments applied to protect soybean from white rot were highly effective, sometimes (Td_{50}) even superior to chemical standards. Efficacy of seed treatments with *Trichoderma* spp. was consistently better than soil treatments with *C. minitans*. Efficacy of seed dressing with Td_{50} was very good for a biological product, its values ranging between 71.7 and 91.7%, superior to chemical standards (62.4-86.2%). The effectiveness of Td_{50} applications resulted in diminished white rot attack frequency in this variant (F% = 1.2-5.8), compared to the untreated control (F% = 8.5-20.5). Likewise, very good efficacy (E% = 74.2-83.0) was obtained with Trichodex 25 WP applied as a seed dressing, consequently lessening frequency of white rot attack (F% = 3.0-5.0), compared to the control (F% = 8.5-20.5). Soil treatment with *C. minitans* also provided good protection to soybean from white rot (E% = 63.4-86.9), the attack frequency in this variant being 1.9-6.3%.

Efficacies reached 62.4-86.2% with chemical standards, with frequency of diseased plants being 2.0-6.2%. Lower efficacy values for chemical standards could be ascribed to the use in some years (1988-1990) of some fungicides devoid of high specificity to *S. sclerotiorum* (AC-8, Tiradin 75 PP).

Soybean seed yield was similar in all years except for 1990, when yields were the lowest. The highest seed yields were recorded with the standard chemical treatment, ranging from 1200 to 2000 kg/ha, and less in 1990 (825 kg/ha), compared to the control (1050-1800 kg/ha, except for 1990, with only 750 kg/ha). *T. viride* (Td_{50}) treatment yielded 1165-1990 kg/ha, and 860 kg/ha in 1990 whilst the seed yield in the Trichodex 25 WP treatment varied between 1175 to 1330 kg/ha during 1991-1994.

Table 2. Efficacy of treatments with *Coniothyrium minitans* (C.m.) and *Trichoderma* spp. (Td_{50}, Trichodex 25 WP) to prevent soybean white rot (*Sclerotinia sclerotiorum*) at Oradea (1988-1994)

Year Cv.	Variant	F %	E %	CE %	Seed yield (kg/ha)
1988	C.m.	1.9	86.9	101.0	1425
Evans	Td_{50}	1.2	91.7	106.0	1475
	AC 8	2.0	86.2	100.0	1520
	Control	14.5	-	-	1350
1989	C.m.	1.9	76.6	120.0	1960
Evans	Td_{50}	1.2	85.9	133.0	1990
	AC 8	3.0	64.7	100.0	2000
	Tiradin	3.2	62.4	96.0	1960
	Control	8.5	-	-	1800
1990	C.m.	6.3	63.4	99.0	840
Evans	Td_{50}	4.7	72.7	114.0	860
	Tiradin	6.2	64.0	100.0	825
	Control	17.2	-	-	750
1991	C.m.	6.2	69.7	99.0	1250
Evans	Td_{50}	5.8	71.7	113.0	1310*
	Trichodex 25WP	5.0	75.6	98.0	1330*
	Sumisclex	4.7	77.0	100.0	1360*
	Control	20.5	-	-	1200
1992	C.m.	4.3	75.4	94.0	1175
Hodgson	Td_{50}	3.8	78.3	97.0	1230
	Trichodex 25WP	3.0	83.0	104.0	1255*
	Sumisclex	3.5	80.0	100.0	1250*
	Control	17.5	-	-	1075
1993	C.m.	3.8	74.7	95.6	1255
Diamant	Td_{50}	3.5	76.7	98.3	1250
	Trichodex 25WP	3.2	78.7	100.9	1260*
	Sumisclex	3.3	78.0	100.0	1270*
	Control	15.0	-	-	1100
1994	C.m.	3.7	69.1	90.1	1140
Hodgson	Td_{50}	3.2	73.3	95.6	1165
	Trichodex 25WP	3.1	74.2	96.7	1175
	Sumisclex	2.8	76.7	100.0	1200*
	Control	12.0	-	-	1050

* Significantly different from the control at $P < 0.05$;
F % - Disease frequency; E % - Efficacy of control;
CE % - Control effect in comparison with standard

C. minitans treatments resulted in seed yields between 1140 and 1960 kg/ha (except in 1990 with 840 kg/ha). In all trials, biological treatments to soil or seed resulted in yield gains from 50 to 190 kg/ha, similar to that of the chemical standard ranging from 75 to 200 kg/ha. Among biological treatments applied, those with *Trichoderma* spp. resulted in higher yield gains, 110 to 190 kg/ha, while *C. minitans* applications gave lower gains, 50-160 kg/ha.

Our previous results with soybean (Şesan & Csép, 1991, 1992, 1993, 1994) as well as those summarized in the present report, represent the first contributions to the use of *C. minitans* as a biological agent in controlling the white rot of this plant (Whipps & Gerlagh, 1992).

This paper reports the first attempts in this country to use *T. harzianum* (T - 39) from the bioproduct Trichodex 25 WP for the biological control of white rot induced by *S. sclerotiorum* in sunflower and soybean in Romania.

The results obtained with biological treatments to soil with *C. minitans*, and to seed with *T. viride* or *T. harzianum* (Trichodex 25 WP) generally demonstrated good protection of sunflower and soybean crops from white rot. However, only occasionally was control equivalent to that afforded by seed dressing with fungicides such as Sumisclex 50 WP which have a more specific activity against *S. sclerotiorum*. Nevertheless, biological treatments provide a possible, environmentally-acceptable solution to protection of these industrial crops from *S. sclerotiorum* to a certain extent.

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INTRODUCTION OF GENES FOR OXALATE DEGRADING ENZYMES INTO PLANTS AS A POTENTIAL MEANS OF PROVIDING TOLERANCE TO *SCLEROTINIA*

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Abstract

Oxalic acid is thought to have a primary role in the pathogenesis of *Sclerotinia sclerotiorum*. A gene coding for the enzyme oxalate oxidase (OxOx) was isolated from barley roots and introduced into oilseed rape as a means of degrading oxalate *in vivo*. This report describes the production of transgenic plants of oilseed rape containing the OxOx gene and the characterisation of these plants by Southern, Western and enzyme activity assays. Plants were shown to contain an active oxalate oxidase enzyme and were tolerant of exogenously supplied oxalic acid.

Introduction

Oxalic acid is produced by a number of plant pathogenic fungi and is thought to play an important role in the pathogenesis of several species, including the wide host-range pathogen *Sclerotinia sclerotiorum* (Noyes & Hancock, 1981; Rowe, 1993). Evidence comes from studies on mutant strains of this fungus, deficient in oxalate production, which are avirulent. Revertants regain their virulence (Godoy *et al.*, 1990).

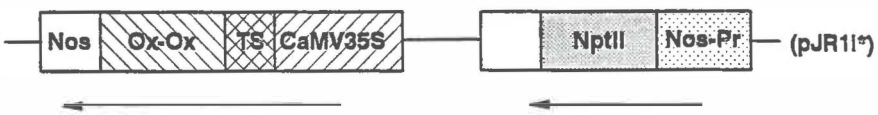
Our aim is to introduce a gene coding for an oxalate-degrading trait into susceptible crop species, then assess the tolerance level of modified plants to pathogens which utilise oxalate in their infection process. There are two known enzymes which can catabolize oxalate, namely oxalate oxidase (EC 1.2.3.4) and oxalate decarboxylase (EC 4.1.1.2). Oxalate oxidase has been isolated from a number of plant species including barley (Chiriboga, 1966). It is considered to be a large oligomeric protein of around 125 kD which can be purified down to a 25kD monomer. Purified preparations of the wheat protein germin also have strong oxalate oxidase activity, associated with the oligomeric (125kD) fraction (Lane *et al.*, 1993). The sequence of the oxalate oxidase gene has also been found to possess close homology to germin (Lane *et al.*, 1993), indicating a possible function for this germination-related protein.

We have made a plant transformation vector containing an oxalate oxidase gene, isolated from a barley root cDNA library and transformed the construct into oilseed rape via *Agrobacterium*-mediated transformation. We outline here the successful expression of oxalate oxidase in the tissues of a range of independent oilseed rape transformants. Readily detectable levels of protein (oxalate oxidase) and enzyme activity (degradation of exogenous oxalate) are reported.

Materials and methods

Vector construction and transformation

A full-length oxalate oxidase clone (710 bp) was constructed from a partial clone obtained from a barley root cDNA library and synthetic oligonucleotides. The clone was attached to a cell wall targeting sequence from *Nicotiana plumbaginifolia* and inserted, with the CaMV35S promoter, into a binary (*pBin19*) *Agrobacterium* vector, to give plasmid *pSR2* (see Figure 1) then re-introduced into *A. tumefaciens* strain LBA4404. The transit peptide was included to target the protein to the extra-cellular space, where the toxin is secreted.



TS - Wall Targeting Sequence * pBin19 derivative

Figure 1. Oxalate oxidase transformation vector pSR2

Cotyledon petioles of oilseed rape cv. Westar were transformed using a modified version of the method of Moloney *et al.* (1989) and transformed tissue selected on the basis of resistance to kanamycin. We have also produced transgenic material containing the oxalate decarboxylase gene.

Molecular and biochemical analysis of transgenics

Rooted plantlets were tested for the presence of the oxalate oxidase gene via standard PCR and Southern analysis. Confirmed transformants were then assayed with Western Blots for presence of the oxalate oxidase protein, using a polyclonal antibody raised to the 25 kD oxalate oxidase monomer.

Oxalate oxidase enzyme activity assays were based on the measurement of reaction products (i.e. H_2O_2 or CO_2). For rapid, non-quantitative assays a membrane-based stain technique was used. Briefly, 300 µg of tissue was ground in an Eppendorf tube with 300 µl water, polyvinyl polypyrollidine and washed sand. Crude extract (50 µl) was spotted onto Hybond C nitrocellulose which was then bathed in developing solution (for 100 ml :- 1mM oxalic acid in 0.1M succinate buffer adjusted to pH 3.5, 20mg 4-chloronaphthol, 0.8 mg horseradish peroxidase). This method gives a readily visible insoluble purple precipitate. For quantitative assays the colorimetric method described by Sugiura *et al.*(1979) which measures

H_2O_2 production, proved more reliable than the ^{14}C labelled oxalate technique (Chiriboga, 1966). These assays can be performed on very crude extracts of small tissue samples.

A combination of the above assays was used to identify homozygous expressing lines, with single locus inserts, by observing segregation patterns.

Excised leaf oxalate wilting test

Excised leaves of oilseed rape plants wilt rapidly when exposed to oxalate solutions. To assess biological activity of the oxalate oxidase enzyme, small photosynthetically active leaves were cut from transformed and control plants and the petioles placed into 20 mM oxalate solutions prepared at pH 4 or water (pH 4) as control. The leaves were observed for signs of wilting after 2-24 hours.

Results

Transformation and molecular analysis of transgenics

A total of 19 independent *pSR2*-transformed plants were generated. When transferred to soil in growth rooms, most appeared phenotypically normal. Southern and PCR analysis of a range of putative transformants revealed very few escapes. Most of the plants tested had a single site of integration, but a few had two or more sites. These were not studied further. Separate shoots derived from the same transgenic callus were usually, but not always, found to be clonal.

Western analysis of transgenics

Western analysis of leaf and root tissue extracts, with a polyclonal antibody raised to the 25 kD monomer of oxalate oxidase, revealed a strongly hybridising band at 25 kD in all transgenics which were PCR and Southern positive. This antibody cross-hybridises faintly to several non-specific bands in control tissue, including a faint band just smaller than 25kD, but a strong 25kD band was never observed in untransformed Westar tissue.

Oxalate oxidase activity assays

All Western positive plants gave positive results when tissue was tested for oxalate oxidase enzyme activity. No activity was observed from control or escape tissue. Quantitation of activity via the spectrophotometric technique gave a range of values for transformed plants from 2 to 47 nmoles H_2O_2/min/mg protein. The assay is subject to significant variability between experiments, but even so, no correlation between gene copy number and expression levels was seen in the primary transgenics. Highly expressing mature plants have activities similar to that detected in barley root material (Chiriboga, 1966). Figure 2 shows the range of activity obtained in a segregating population of plants with a single site of insertion.

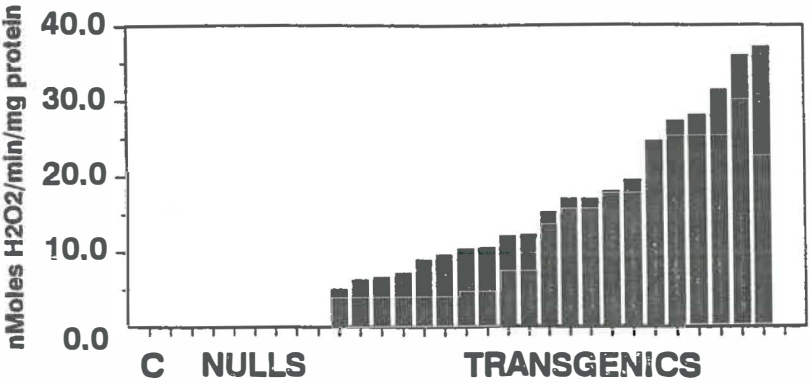


Figure 2. Oxalate oxidase activity in progeny from a primary transgenic plant

Oxalate wilting test

When petioles of excised leaves of control plants were placed into oxalate solutions adjusted to pH 4 they wilted rapidly, most probably due to xylem embolisms caused by the oxalate. Excised leaves fed with pH 4 water remained turgid. Leaves of some of the transgenic lines expressing oxalate oxidase did not wilt in the oxalate solutions, even over an extended 24 hour period. Only three lines, selected on the basis of high levels of expression, were tested in this way and all three showed resistance to wilting.

Plants containing the oxalate decarboxylase enzyme have yet to be analysed fully.

Discussion

The barley oxalate oxidase gene has been successfully introduced into oilseed rape. The gene is constitutively expressed in all tissues of the plant tested so far although some differences in expression levels between different tissues are likely. The gene product (a 25kD monomer) either has oxalate oxidase activity itself, or is correctly processed in the cell to form a re-associated oligomer, giving an active enzyme. Further tests are underway to determine the exact form of the enzyme and its localisation in the transgenic plants. We have observed the biological action of the oxalate oxidase enzyme in transgenic plants, as relates to protection against wilting of excised leaves dipped in an oxalate solution. However, the value of these transgenic plants as a source of disease resistant breeding material depends upon extensive disease screening, not only under laboratory conditions, but more importantly in the field. Such trials are now underway.

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GROWTH AND TRANSMISSION OF *CONIOTHYRIUM MINITANS* IN SOIL

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Abstract

In laboratory tests, using *Coniothyrium minitans*-infected sclerotia of *Sclerotinia sclerotiorum* placed on sterile or non-sterile soil, mycelial extension of *C. minitans* was only observed on sterile soil, indicating that spread by direct mycelial growth in natural soil is unlikely. The role of soil mesofauna in the spread of *C. minitans* was also studied using the collembolan *Folsomia candida* and the mite *Acarus siro*. Both *F. candida* and *A. siro* transmitted the mycoparasite to uninfected sclerotia in sterile and non-sterile soil adjusted to a range of water potentials (-0.25 to -3.6 MPa). These results suggest that soil mesofauna may be important in the horizontal spread of *C. minitans*.

Introduction

Coniothyrium minitans Campbell is a mycoparasite of sclerotia, and has been found mainly in those of the plant pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary (Whipps & Gerlagh, 1992). Little is known about the ecology or mechanisms of dissemination of *C. minitans*, and its isolation from soil by dilution plating is rare (Whipps *et al.*, 1993). The fungus can survive for extended periods in soil within infected sclerotia, but whether it can grow on other organic material in the soil is not known (Whipps & Gerlagh, 1992). A single report (Magan & Whipps, 1988) suggests that *C. minitans* is unable to grow through soil but this has not been substantiated.

During glasshouse trials on the use of soil applications of *C. minitans* for biocontrol, it was observed that the mycoparasite spread to untreated control plots (Budge & Whipps, 1991). The presence of numerous mites and collembolans associated with *S. sclerotiorum*-infected plant debris from these trials led to speculation that soil fauna may be important in the dissemination of *C. minitans* in soil. There is some evidence that soil fauna may play a role in the dispersal of *C. minitans* (Trutmann *et al.*, 1980; Whipps & Budge, 1993) but this has not been fully investigated.

In this paper, the growth of *C. minitans* from live and autoclaved infected sclerotia on sterile and non-sterile soil is described. Also, preliminary data are reported on the effect of different water potentials on the ability of the mycophagous collembolan *Folsomia candida* Willem, and the polyphagous astigmatid mite *Acarus siro* L., to transmit viable propagules of *C. minitans* to uninfected sclerotia in sterile and non-sterile soil.

Materials and Methods

Maintenance of fungi, collembolans and mites

Sclerotinia sclerotiorum isolated from infected lettuce tissue and *C. minitans* (IMI 134523) originally isolated from a sclerotium of *S. sclerotiorum* were maintained on potato dextrose agar (PDA) (Oxoid, UK) at 18-20°C. Sclerotia for experimental use were produced by PDA culture or wheat grain culture. Sclerotia from PDA cultures were collected when firm and dry, approximately 4 weeks after inoculation. Sclerotia from wheat grain culture were collected and air-dried overnight, 3 weeks after inoculating flasks containing 25 g of wheat grain that had previously been autoclaved in 50 cm^3 de-ionised water (Mylchreest & Wheeler, 1987). All sclerotia for experimental use were selected for 2-4 mm diam. and used within 4 weeks. Where necessary, infection of sclerotia with *C. minitans* was achieved by placing them on the periphery of *C. minitans* PDA cultures for 10 days.

The collembolan *F. candida* was maintained on PDA cultures of *C. minitans* (Whipps, 1993). The astigmatid mite *A. siro* was cultured in a 3:1 mixture of yeast (Yesta 20B dried yeast, CPC Ltd, UK) and wheatgerm (Bemax Wheatgerm, Boots Ltd, UK) (George, 1982).

Sterilisation and moisture adjustment of soil

Brickearth soil (silt loam, Hamble series) from HRI Littlehampton was collected, passed through a 2 mm sieve, air dried and stored in sealed polyethylene bags at 10°C and used within 4 weeks. Soil water potentials were adjusted to between -0.2MPa (moist) and -7.2MPa (relatively dry) by the addition of appropriate quantities of water, using the moisture adsorption curve determined for this soil by Magan *et al.* (1989). Subsequently, the required water potentials were maintained by the weekly addition of sterile water. Between weekly adjustments, water loss never exceeded 5% of the total initially added to the soil.

Soil was sterilised by autoclaving (122°C, 15 min) 25 g quantities on two consecutive days. The soil was left for 24-48 h before use to allow any volatile inhibitory compounds released by autoclaving to dissipate. Prior to use, soil sterility was checked by plating samples onto PDA and nutrient agar (NA) (Oxoid, UK).

Growth of C. minitans *in soil*

The ability of *C. minitans* to grow in soil was investigated using a modified soil sandwich technique (Grose *et al.*, 1984). A nylon membrane (Whatman No. 7402004, 47 mm diam. filter) was placed on the surface of a layer of soil in a 90 mm diam. Petri plate adjusted to -0.2, -3.6 and -7.2MPa water potential. A single sclerotium, either infected with *C. minitans* or autoclaved and infected with *C. minitans,* was then placed on the centre of the membrane. The plates were incubated in the dark at 18-20°C. Plates were periodically examined and samples of mycelium growing from the sclerotia were plated onto PDA + chlortetracycline (0.02 g $litre^{-1}$ powder containing 80% chlortetracycline HCl, Sigma Chemicals, USA) for identification. Three replicate plates of each sclerotium treatment were set up, and the experiment was repeated using non-sterile soil.

Transmission of C. minitans *by collembolans and mites*

For these studies, sterile or non-sterile soil adjusted to -0.25 or -3.6 MPa water potential was placed in 140 mm diam. Petri plates. Three wheat grains were then placed in the centre of the Petri plates. These grains had previously been autoclaved and infected with *C. minitans* by placing them on the periphery of PDA cultures of the fungus for 2 weeks. To each plate was added a ring of 5 sclerotia placed 55 mm from the centre of the inoculum source, and 20-30 collembolans or 200-300 mites. Control plates lacking an inoculum source and/or lacking the insects/mites were also set up. Plates were incubated in the dark at 18-20°C. After 2, 4 and 6 weeks the plates were examined and the sclerotia collected, surface sterilised, bisected and placed on plugs of PDA + chlortetracycline (Whipps & Budge, 1990) to check for the presence of *C. minitans*. The experiment was replicated 4 times with collembolans and 3 times with mites. All replicates were set up at different times.

Results and Discussion

Growth of C. minitans *in soil*

Mycelial growth of *C. minitans* from infected sclerotia was not observed on non-sterile soil during the time-scale of this study (7 weeks). This is in agreement with the results of Magan & Whipps (1988) and suggests that the mycoparasite is unable to spread by mycelial extension in natural soil. Moreover, both live and autoclaved *C. minitans*-infected sclerotia rapidly became colonised by other fungi from the soil, which is consistent with the description by Tribe (1957) of the secondary invasion of *C. minitans*-infected sclerotia by soil microflora.

On moist sterile soil (-0.2 MPa water potential) *C. minitans* grew from both live and autoclaved infected sclerotia, forming characteristic pycnidia on the membranes. On drier soil (-3.6 MPa and -7.2 MPa), *C. minitans* grew only from live infected sclerotia. Reasons for this difference are being investigated.

Transmission of C. minitans *by collembolans and mites*

Both mites and collembolans were able to transmit *C. minitans* to uninfected sclerotia on sterile and non-sterile soil (Table 1). Isolation of *C. minitans* from sclerotia from non-sterile controls lacking added fauna and an inoculum source of the mycoparasite was extremely rare (2 out of 840 sclerotia sampled; data not shown). This indicates that the background level of *C. minitans* in the soil used was negligible, and cross contamination infrequent. Similarly, very few sclerotia from non-sterile controls lacking added fauna, but with a source of *C. minitans* inoculum, were infected with the mycoparasite. This supports the observation made earlier, that the fungus does not spread through non-sterile soil myceliogenically, and suggests that any indigenous fauna in the soil used for this study had no significant effect on the spread of *C. minitans*. In control plates consisting of sterile soil with a source of *C. minitans* inoculum, but lacking added fauna, the mycoparasite spread myceliogenically through both moist and relatively dry soil to infect significant numbers of sclerotia. However, the addition of either collembolans or mites greatly increased spread of the mycoparasite on sterile soil.

Table 1. Transmission of *C. minitans* from inoculated wheat grains to sclerotia of *S. sclerotiorum* by *F. candida* and *A. siro* incubated for 6 weeks on sterile and non-sterile soil at 18-20°C

Added fauna	Water potential (MPa)	Number (%) of sclerotia infected with *C. minitans*			
		Sterile soil		Non-sterile soil	
		Without added fauna	With added fauna	Without added fauna	With added fauna
F. candida[a]	-0.25	4 (20)	17 (85)	0 (0)	4 (20)
	-3.60	2 (10)	4 (20)	0 (0)	1 (5)
A. siro[b]	-0.25	1 (7)	14 (93)	0 (0)	5 (33)
	-3.60	10 (67)	15 (100)	0 (0)	15 (100)

Each Petri dish contained 5 uninfected sclerotia at the beginning of the experiment, giving: [a] 20 sclerotia in total from 4 replicates; [b] 15 sclerotia in total from 3 replicates.

On non-sterile soil, collembolans transmitted the mycoparasite most effectively on moist soil (-0.25 MPa), with 20% of sclerotia infected by *C. minitans* after 2 weeks (data not shown). Longer incubation had no effect. Mites transmitted the mycoparasite most effectively on relatively dry soil (-3.60 MPa), with 93% of sclerotia infected after only 2 weeks, rising to 100% after 6 weeks.

These results are in agreement with those of Whipps & Budge (1993), in demonstrating that collembolans are capable of transmitting viable spores of *C. minitans* to uninfected sclerotia in non-sterile soil. They are also consistent with the results of Griffiths *et al.* (1959) in showing that *A. siro* can disseminate fungal spores. Soil moisture, an important factor in the ecology of soil arthropods (Wallwork, 1976), clearly influenced the transmission of *C. minitans* by *F. candida* and *A. siro* in this study. However, these results suggest that, over a range of moisture contents, soil mesofauna may play an important role in the dissemination of *C. minitans*.

Acknowledgements

This work was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and the Ministry of Agriculture Fisheries and Food (MAFF) for England and Wales. We thank Dr R.C. Cooke for many valuable discussions.

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ASPECTS OF PARASITISM OF SCLEROTIA-FORMING FUNGI BY *CONIOTHYRIUM MINITANS*

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Abstract

An attempt has been made to culture *Coniothyrium minitans* on living sclerotia of *Sclerotinia sclerotiorum*, *S. minor* and *Botrytis cinerea* placed on water agar. Sclerotia of *S. sclerotiorum* and *S. minor* were parasitized *in vitro*, but sclerotia of *B. cinerea* were not. Parasitism of hyphae of the pathogens has also been studied and coiling and penetration were observed as typical signs of parasitism. This was commonly recorded on hyphae of *S. sclerotiorum*, *S. minor* and *B. cinerea*.

Since there are no records in the literature on parasitism of sclerotia of *S. minor* by *C. minitans in vivo*, sclerotia of *S. minor* were added to soil pre-inoculated with conidia of *C. minitans* in diatomaceous earth. After an incubation period of three weeks, a first sample of sclerotia was screened for infection by *C. minitans*. Sclerotia were washed, placed on a slide, and microscopically observed while pressing the sclerotia with a needle. Huge amounts of conidia of *C. minitans* were released by infected sclerotia. Sampling was repeated twice at weekly intervals. Most of the sclerotia showed infection.

Introduction

Coniothyrium minitans Campb. was first described when isolated from sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary in the U.S.A., and its potential for biological control of this pathogen was noted immediately (Campbell, 1947). The mycoparasite has been recorded in many countries since. Parasitic activity on sclerotia and hyphae of *S. sclerotiorum* and *S. minor* Jagger was noticed by many workers. Details of hyphal interactions between *C. minitans* and *S. sclerotiorum* have been elucidated using both light and electron microscopy (Huang & Hoes, 1976; Tu, 1984; Huang & Kokko, 1988). Hyphal interactions also have been studied in dual cultures. On PDA, *C. minitans* commonly inhibits colony growth of a range of pathogens slightly before contact and then continues to overgrow, and in some cases such as *S. sclerotiorum* and *Sclerotium cepivorum* Berk., destroys hyphae of the pathogen (Ghaffar, 1972; Zazzerini & Tosi, 1985; Harrison & Stewart, 1988; Phillips, 1989; Whipps & Gerlagh, 1992). Detailed microscopical observations of infection of sclerotia have only been carried out with *S. sclerotiorum* and *S. cepivorum* (Phillips & Price, 1983; Tu, 1984; Huang & Kokko, 1987; Stewart & Harrison, 1988; Whipps & Budge, 1990). Therefore, we studied the changes in hyphae and sclerotia of *S. sclerotiorum*, *S. minor* and *Botrytis cinerea* Pers. ex Nocca & Balb. during parasitism by *C. minitans*.

Materials and Methods

The hyphal interactions and the parasitism of *S. sclerotiorum*, *S. minor* and *B. cinerea* by *C. minitans* were studied using methods of Ikediugwu & Webster (1970) and Ikediugwu *et al.*, (1970), modified by Skidmore & Dickinson (1976). Small blocks of agar with fungal colonies from the margin of vigorously growing young cultures were placed approximately 2 cm apart on cellophane over potato dextrose agar (PDA). The plates were incubated at 25 ± 2°C. The colonies met after 3 days incubation and were then examined under the microscope. For detailed observations, small squares of cellophane were cut from the area of intermingling growth. These squares were mounted on microscope slides and were observed after staining with cotton blue.

The parasitic activity of *C. minitans* on individual sclerotia was tested on water agar. Sclerotia were distributed over a Petri dish and each sclerotium was inoculated with a 2 mm^2 piece of agar from the margin of a culture of *C. minitans* on PDA. After 3 weeks incubation at about 25°C, sclerotia were examined microscopically.

Infection of sclerotia of *S. minor* by *C. minitans* was also examined in a sandy loam soil. Soil (100 g) was inoculated with 1 g diatomaceous earth, containing about 2 x 10^6 conidia of *C. minitans* per g. Inoculated soil was then placed in 15 cm diam. pots and three nets, each containing 30 sclerotia of *S. minor*, were placed 5 cm deep in each pot. After three, four and five weeks of incubation at ± 25°C, infection was determined by microscopic examination of the sclerotia from one net. Sclerotia were washed, placed on a microscope slide, and pressed with a needle. Release of conidia from pycnidia of *C. minitans* was proof of infection.

Results and Discussion

In dual cultures the antagonist *C. minitans* grew over the colonies of *S. sclerotiorum*, *S. minor* and *B. cinerea*. Parasitism of the hyphae of *S. sclerotiorum*, *S. minor* and *B. cinerea* by *C. minitans* was very common. It was initiated by direct contact and coiling of the mycoparasite around the host hyphae. According to Dennis & Webster (1971) coiling is not solely due to the contact stimulus, but some unknown factors may also be involved. The hyphae running over the host are branched and adhere to the host hyphae, coiling around them and forming penetration pegs. Penetration of host hyphae and also of the conidia of *B. cinerea* by the parasite was observed. After penetration, the parasite developed modified, wavy mycelium which usually pervaded the hyphae of the host, passing through the septa. Moreover, the hyphae of *S. sclerotiorum* were observed to lyse subsequent to coiling and penetration by *C. minitans*. It was evident that only the parasitizing hyphae were coloured by cotton blue stain. Lysis and disruption of protoplasm was also observed in non-contacted hyphae. These changes may be due to the effect of diffusing antibiotics produced by the parasite. The adverse effect of the parasite on the host is presumably due to the physiological changes (Dennis & Webster, 1971) brought about by the action of the parasite on the host metabolism.

The size of the host hyphae could also be a physical barrier in penetration phenomena. It was postulated by Dennis & Webster (1971) that wider hyphae were easily penetrated by the narrower ones. This was also found to be true in our present investigation.

On the basis of the nutritional relationship of the parasite with the host, Barnett & Binder (1973) classified parasitism into two main groups, necrotrophic and biotrophic. Coiling and penetration (Barnett & Lilly, 1962; Dennis & Webster, 1971) are common to both interaction types, but degradation of cytoplasm and bursting of the hyphae (Skidmore & Dickinson, 1976) are typical of a necrotrophic relationship.

Mycoparasitic activity of *C. minitans* on sclerotia of *S. sclerotiorum*, *S. minor* and *B. cinerea* was examined *in vitro*. Sclerotia of *S. sclerotiorum* and *S. minor* were parasitized but sclerotia of *B. cinerea* were not. Microscopic observation of the development of *C. minitans* on sclerotia of *S. minor* revealed that the mycoparasite produces pycnidia on the surface of the sclerotia. Microscopic examination of free hand sections of infected sclerotia indicated that the mycoparasite invaded the medulla of sclerotia of *S. sclerotiorum* and *S. minor* as evidenced by the presence of the blue stained hyphae of *C. minitans* at various locations within the hyaline sclerotial tissue. In the case of *S. sclerotiorum*, several pycnidia developed inside the sclerotia.

Infection of sclerotia of *S. minor* placed in sandy loam soil inoculated with *C. minitans* began within 3 weeks. Huge amounts of conidia oozed out of sclerotia placed on a slide when pressed by a needle.

The infection and destruction of sclerotia of *S. minor in vivo* has not been recorded so far. The infection of sclerotia brought about by *C. minitans* introduced into a natural soil indicates that this mycoparasite has considerable potential as a biological control agent by reducing the inoculum density of *Sclerotinia* spp. Studies to determine the effectiveness of *C. minitans* in biological control are in progress.

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A UNIQUE BACTERIAL DISEASE OF *CONIOTHYRIUM MINITANS*

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Abstract

Several isolates of *Coniothyrium minitans* from sclerotia of *Sclerotinia sclerotiorum* exhibited abnormal growth on agar media. These isolates apparently contained bacteria which could not be isolated and cultured *in vitro*. Inclusion of antibiotics (amicacin, chloramphenicol, garamicin, penicillin, streptomycin or tetracycline) in the media failed to kill the bacteria within the mycelium of *C. minitans*. The identity of the bacteria is unknown.

Introduction

Chemical control of plant diseases caused by sclerotial pathogens has not been satisfactorily achieved in most cases. The major difficulties arise from the longevity of sclerotial populations in soil, the ability of many sclerotial pathogens to attack their hosts over prolonged periods, the below ground infection court of many sclerotial pathogens, and the cost and limited effectiveness of attempts to eradicate sclerotia chemically from soil and crop debris (Coley-Smith & Cooke, 1971). In view of the great number of papers on chemical control of sclerotial fungi which have accumulated over more than 20 years, the problems still seem to be unsolved (Ahmed & Tribe 1977; Adams, 1989, 1990).

Biological control may offer a sustainable method for the agricultural management, if a suitable agent, the cost of application and the essential ecological factors are known. Examining the literature on *Coniothyrium minitans*, it seems that this mycoparasite may be a useful tool against several sclerotial fungi in future biocontrol programmes. *Coniothyrium minitans* can be cultured and detected from different types of soil far more easily than another promising mycoparasite *Sporidesmium sclerotivorum* (Litkei, 1990). The capacity of *C. minitans* to degrade sclerotia is significantly better than *Gliocladium* spp., *Trichoderma* spp., *Nectria inventa*, but it is similar to *S. sclerotivorum* (Litkei, 1990).

Besides these useful characteristics there may be some limiting factors in the development of *C. minitans* as a biocontrol agent. This paper reports on the occurrence of a novel bacterial disease of *C. minitans* although the bacteria involved have not been identified to date.

Materials and Methods

Numerous overwintered sclerotia of *Sclerotinia sclerotiorum* were collected from a sunflower field in May 1993 near Szarvas in Hungary. The sclerotia were surface sterilised and placed in Petri dishes (10 per dish) containing moist sterile sand (pH 6.5), and were incubated for 10 days at 20°C. Several isolates of *Coniothyrium minitans* were subsequently obtained from the sclerotia and some exhibited abnormal colony morphology on agar and contained an

unknown bacterium. The abnormal isolates of *C. minitans* were then placed in Petri dishes containing 20 ml PDA containing 1, 5, 10, 15 mg l^{-1} of the following antibiotics: penicillin, streptomycin, tetracycline, amicacin, garamicin, chloramphenicol. The Petri dishes were incubated for 10 days at 22°C and growth monitored. The abnormal isolates of *C. minitans* were compared with a healthy isolate of *C. minitans* provided by Dr. M. Gerlagh, Research Institute for Plant Protection, Wageningen.

Results

Morphological abnormalities were observed in some *C. minitans* isolates growing on PDA. The characteristics of a representative isolate (*Coniothyrium minitans* 5), include: a change in pigmentation of the pycnidia with some pigment appearing in the PDA; a decrease in the number of the pycnidia produced; production of deformed pycnidia, being frequently collapsed and almost empty; limited pycnidiospore production; occasional production of microconidia; increased hyphal diameter and increased mycelial growth. In addition, some abnormal hyphal cells were full of bacteria, some were empty or contained very few bacteria; bacterial movement in the full cells was much greater than in those cells where numbers of bacteria were few; the bacteria accumulated particularly at the septa of the abnormal cells and not the walls of the cells.

The bacteria were spherical and could not be cultured on any artificial media tested so far. The bacteria could not be identified in the laboratory of Szarvas or the Biological Department of Medical and Biological School of Patras in Greece. In an attempt to cure the mycelium of the abnormal *Coniothyrium minitans* 5 of the bacteria, some antibiotics were incorporated into the agar medium. With amicacin, garamicin, penicillin or streptomycin in the medium, *C. minitans* 5 grew faster than the healthy control (see Figure 1 as an example), but the bacteria within the hyphae were not killed. With tetracycline or chloramphenicol in the medium, growth of *C. minitans* 5 was slower than the healthy mycelia in some tests (Figure 2 and Figure 3) and the number of bacteria present within hyphal cells was reduced.

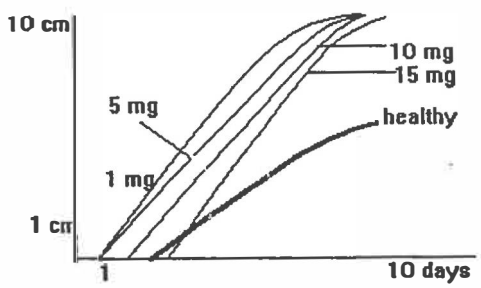

Fig. 1. The growth of the abnormal isolate *Coniothyrium minitans* 5, on PDA containing 1, 5, 10, 15 mg l^{-1} amicacin

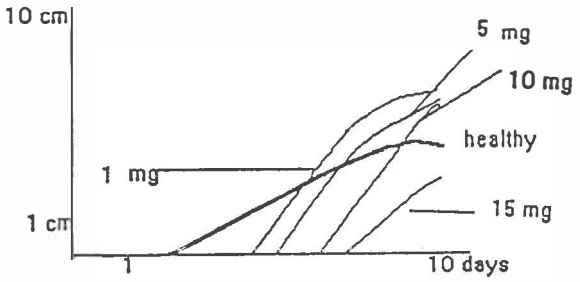

Fig. 2. The growth of the abnormal isolate *Coniothyrium minitans* 5, on PDA containing 1, 5, 10 15 mg l^{-1} tetracycline

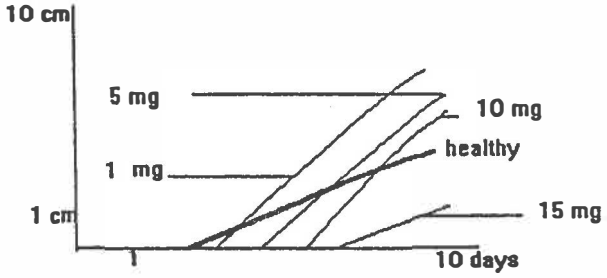

Fig. 3. The growth of the abnormal isolate of *Coniothyrium minitans* 5, on PDA containing 1, 5, 10, 15 mg l^{-1} chloramphenicol

Discussion

To enable us to understand what happens to plant pathogens and their hyperparasites in soil, many microbial interactions need to be understood. Important details of the aetiology of *Sclerotinia sclerotiorum* are known, but similar information on *Coniothyrium minitans* is still not available. It is almost certain that one or more propagules of *C. minitans* are present in soil at all times but where they originate from and how they survive is not clear. Similarly, the kinds of diseases which limit the individual number of *C. minitans* is unknown. Answers to these will be found by studying the ecology of the biocontrol agent (Phillips, 1985; Gerlagh & Vos, 1991; Whipps & Gerlagh, 1992). There are some formulations of mycoparasites already available to growers in different countries for use as biocontrol agents. It is important to know the environmental conditions where the biocontrol agent is applied. There have been some suggestions to mix two strains of a single hyperparasite or two different hyperparasites to apply against the sclerotial fungi. To control the quality characteristics of a composition will be indispensable in the future.

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INFLUENCE OF ORGANIC COMPOSTING SYSTEMS ON SURVIVAL OF SCLEROTIA OF *SCLEROTINIA TRIFOLIORUM* AND *S. SCLEROTIORUM*

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Abstract

Composts with varying combinations of straw and manure were produced and material from the different fermentation phases of the composting process was tested for its anti-phytopathogenic potential. Material from both thermophilic and mesophilic phases influenced the survival of sclerotia of *Sclerotinia trifoliorum* and *S. sclerotiorum*. Exposure of sclerotia for 20 days to material from the thermophilic phase resulted in high levels of pathogen mortality. The mortality was mainly caused by temperatures ranging from 45° to 80°C. Sclerotia mortality during exposure to material from the mesophilic phase was related to the activity of antagonistic microorganisms such as species of *Trichoderma, Gliocladium* and *Chaetomium*.

Introduction

The value of composts, as a substitute for peat and as a substrate to increase soil fertility is well known in horticulture (Gottschall, 1988). The need for the recycling of household wastes has led to concerns about the presence of microorganisms toxic to humans or pathogenic towards plants. A number of publications over the last 20 years have dealt with the survival or mortality of viruses, bacteria, fungi, nematodes and weed seeds in composted organic material (Hoitink & Fahy, 1986). The results indicated that the organisms behave differently during the two phases (thermophilic and mesophilic) of the composting process. Therefore, each pathogen needs to be tested separately for ability to survive. In our investigations we tested the influence of composting systems and the addition of different types of manure on the survival of sclerotia of *Sclerotinia trifoliorum* and *S. sclerotiorum*.

Materials and Methods

A number of different substrates were used to investigate the effect of compost on the mortality of sclerotia.

1. Field soil = control
2. Straw compost (GSK) containing 70% clover and 30% wheat straw
3. Manure compost (GMK) containing 45% clover, 35% horse manure, 15% wheat straw and 5% fermented compost

Sclerotia produced on carrot pieces were placed into the composts at two depths (10 cm (T1) and 30 cm (T2)) in nylon nets (10-15 sclerotia/net). During the thermophilic phase of composting the survival of sclerotia was checked 3, 5, 7, 10, 14 and 20 days after incorporation into the substrates. During the following maturing mesophilic phase, the sclerotia were incorporated into 3 to 24 month-old substrates and removed after 10, 20, 40 or 60 days. Sclerotia were stained with fluorescein diacetate and observed under the fluorescence microscope for viability (Dittmer & Weltzien, 1990).

Results and Discussion

During composting, temperatures in the thermophilic phase regulate survival or mortality of microorganisms. Temperatures in straw compost (GSK) never exceeded 45°C during the summer season (Fig. 1). However, temperatures in the GMK-substrate rose to 80°C in the thermophilic phase (Fig. 2). Comparing the two substrates the results showed that sclerotia mortality of both fungi was greater in the deeper zone of 30 cm (T2) of GMK than in GSK (Fig. 3). Twenty days after being incorporated into the substrates no sclerotia of *S. sclerotiorum* survived in GMK, 10-30% in the GSK and 60-75% in the control soil (Fig. 3). The results of temperature on the mortality of sclerotia during the thermophilic phase correspond to findings of Hoitink & Fahy (1986) and Herrmann *et al.* (1994).

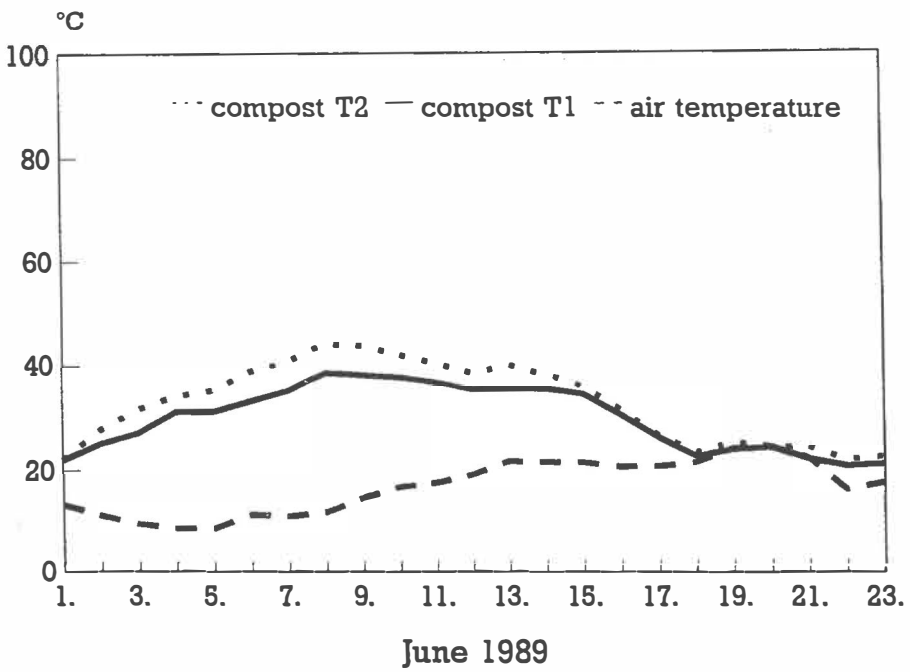


Fig. 1. Temperature during thermophilic phase of composting clover straw (GSK) (T1 = 10 cm deep, T 2 = 30 cm deep)

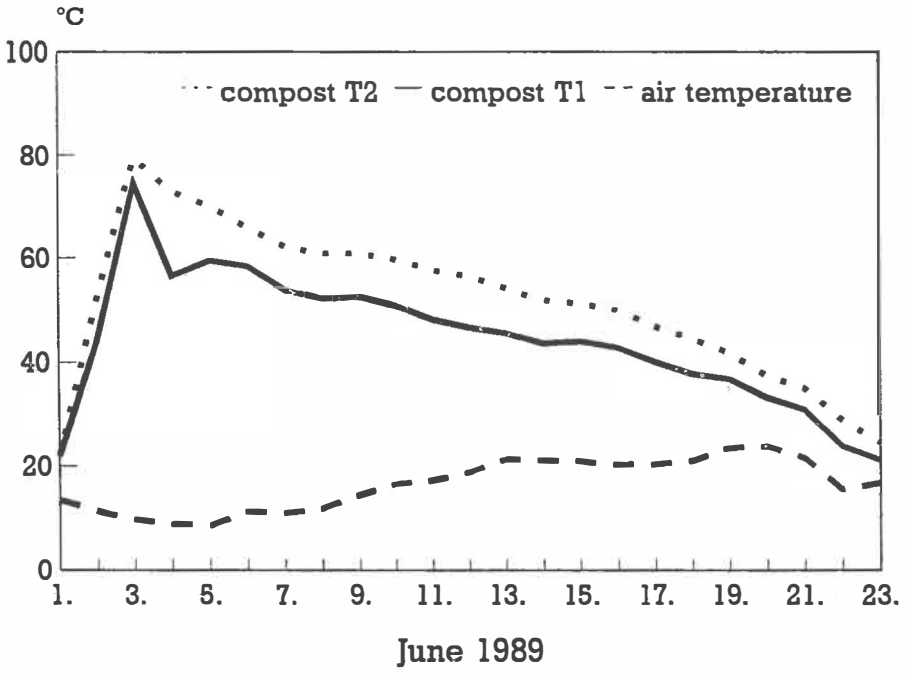
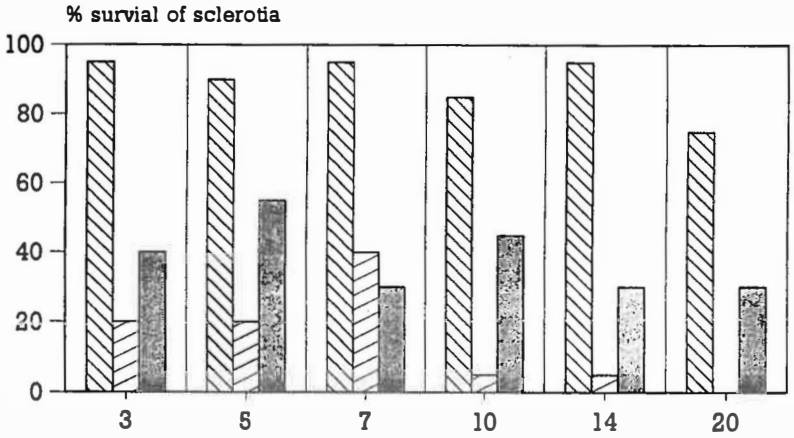


Fig. 2. Temperature during thermophilic phase of composting clover manure (GMK) (T1 = 10 cm deep, T 2 = 30 cm deep)

During the mesophilic phase the viability of sclerotia shows different patterns. For instance, in a 7 month-old compost, sclerotia of *S. trifoliorum* were still viable 10 days after incorporation (Fig. 4). In GSK after 60 days, all the sclerotia survived. However, the mortality rate increased significantly after being buried in GMK for 20-40 days. A higher population of hyperparasitic and antagonistic fungi of sclerotia were isolated from composts containing manure than from other substrates used in the experiments (Dittmer, 1991; Dittmer & Weltzien, 1988). The antiphytopathogenic potential of composts is active in younger composts and is affected by the composition of the substrate. The addition of manure extends suppressiveness effects on the viability of sclerotia.

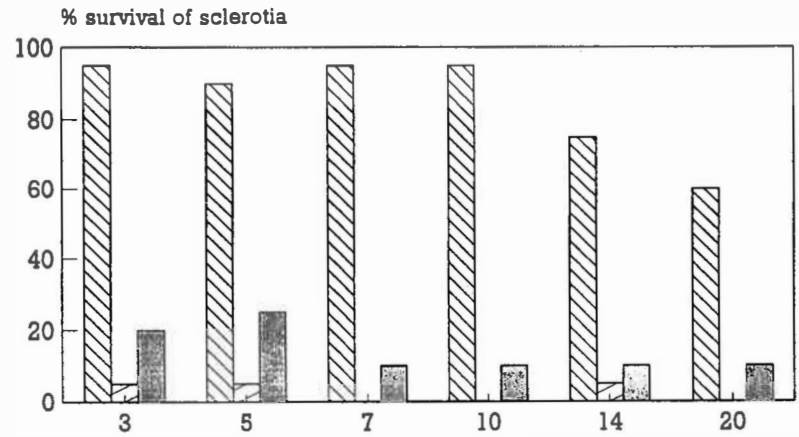
A)



Erde	95	90	95	85	95	75
GMK	20***	20***	40***	5***	5***	0***
GSK	40***	55 *	30***	45 *	30***	30 *

deposition in days ▨ Erde ▨ GMK ▨ GSK

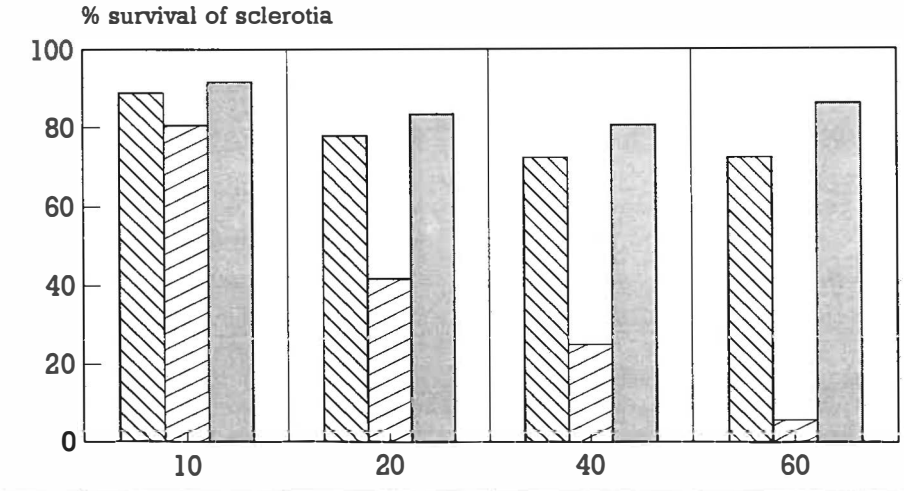
B)



Erde	95	90	95	95	75	60
GMK	5***	5 ***	0 ***	0***	5***	0***
GSK	20***	25***	10***	10***	10***	10 **

deposition in days ▨ Erde ▨ GMK ▨ GSK

Fig. 3. Survival rate of sclerotia of *S. sclerotiorum* buried in soil (Erde), clover manure (GMK) and clover straw compost (GSK) during the thermophilic stage of composting. A): 10 cm (T 1), B): 30 cm (T 2)



Erde	88,89	77,78	72,22	72,22
GMK	80,56	41,67 **	25 ***	5,56 ***
GSK	91,67	83,33	80,56	86,11

deposition in days Erde GMK GSK

Fig. 4. Survival rate of sclerotia of *S. trifoliorum* placed in clover manure (GMK) and clover straw (GSK) during the mesophilic phase of composting, and in field soil (Erde)

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PROSPECTS OF INTEGRATED CONTROL OF LETTUCE DROP CAUSED BY *SCLEROTINIA* SPP.

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Abstract

Field trials involving solarization or solarization combined with a *Trichoderma* amendment, showed solarization to be highly effective against lettuce drop, the best results being comparable with those obtained with standard fungicide treatments with dicarboximides. Efficacy against *Sclerotinia* spp was correlated with total solar radiation during solarization ($r^2 = 0.78$). In climatic conditions of Roussillon (Southern France), 1150 MJ/m^2 seemed necessary to reach the threshold of 50% effectiveness. Equivalent levels of control could be obtained either by a long duration of solarization or a moderate length of solarization immediately followed by an application of a *Trichoderma harzianum* preparation. This could be a means to reduce the duration of solarization. Enhanced biodegradation of dicarboximides (currently the fungicides most frequently used to control lettuce drop) was appreciably reduced by solarization. Consequently, prospects of Integrated Pest Management (IPM) of lettuce drop caused by *Sclerotinia*, combining solarization, *Trichoderma* release and, if necessary, reduced numbers of fungicide applications, look very promising.

Introduction

The problem of loss of field efficacy of cyclic imides (dicarboximides) to control lettuce drop caused by *Sclerotinia* has risen rapidly since 1985 (Martin & Davet, 1986; Walker *et al.*, 1986). Based on the results of Katan (1981) and Porter *et al.* (1988), we have tested solarization as an alternative control measure in intensive lettuce crop production in Southern France since 1989. We also tried to combine solarization with the application of an antagonist such as *Trichoderma harzianum*.

Materials and Methods

We checked the efficiency level of solarization on lettuce drop by *S. minor* by Fischer's complete block protocol, using plots of 100 m^2 with 3 to 4 replicates. The efficiency percentage was calculated according to an Abott type formula, comparing the death rates in the solarized and the non-solarized plots.

$$\% \text{ efficacy} = \left\{ 1 - \frac{\% \text{ D solarized}}{\% \text{ D non-solarized}} \right\} \times 100$$

% D: % dead lettuce due to Sclerotinia (mean of four to five data/trial)

The total solar radiation during solarization was assessed by a Kipp & Zonen pyranometer located in Alenya not further than 10 kilometers from each trial. *Trichoderma* populations were enumerated on the medium of Davet (1979).

Results and Discussion

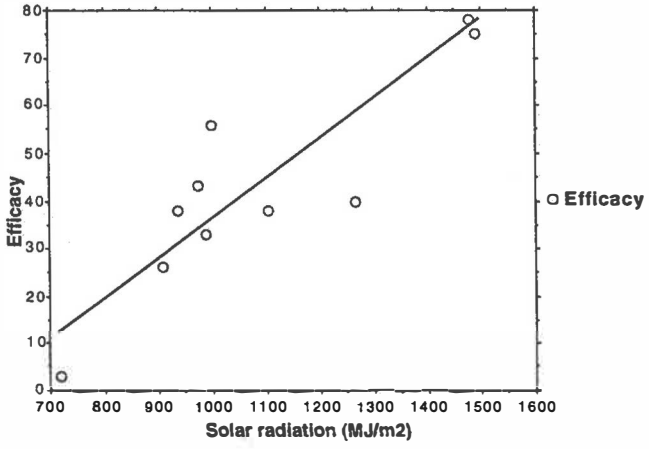

Fig. 1. Effect of increasing solar radiation during solarization on % efficacy of control of *Sclerotinia* on lettuce ($y = 0.08x - 41.94$; $r^2 = 0.78$)

We found a good correlation ($r^2 = 0.78$) between the total solar radiation and the percentage efficacy of control of lettuce drop. A mean value for total solar radiation of 1150 MJ/m^2 achieved 50% efficiency: an important reduction of the risk. In Southern France, the 50% threshold value could be reached in 60 days within a period extending from June 15th to September 10th based on 20 years climatic statistics. These results confirmed the practical potential of solarization for controlling *Sclerotinia* lettuce drop already demonstrated in Australia by Porter *et al.* (1988).

When *Trichoderma* inoculum was introduced into solarized soils, population levels (determined 3 months later) were much higher than the levels obtained in non-solarized soils with the same amount of inoculum. There were 18.5, 29, 24, 61 and 60 x 10^3 cfu/g in solarized soils, compared to 9.5, 13, 12, 16 and 8.5 x 10^3 cfu/g in non-solarized soils, respectively, in 5 different trials. The difference persisted at least one year.

Moreover, solarization slowed down the phenomenon of enhanced biodegradation of

cyclic imides to between 20 to 84% less than that in non-solarized soil in 7 different field trials (Davet & Martin, 1993).

These observations have already influenced growers in Southern France who have now adopted an IPM type approach of lettuce cultivation (Martin & Thicoipe, 1994), following these recommendations:

- solarization of the fields every 2/3 years
- introducing antagonistic fungi after solarization
- reducing number of fungicide sprays (iprodione).

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POTENTIAL FOR CONTROL OF *SCLEROTINIA SCLEROTIORUM* (LIB.) DE BARY IN TOMATO IN NON-HEATED GREENHOUSES BY SOIL SOLARIZATION

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Abstract

Soil solarization in a non-heated greenhouse for 8 weeks during the summer using a 0.05 mm thick transparent polyethylene sheet gave 100% control of stem and fruit rot of tomato caused by *Sclerotinia sclerotiorum*. The number of sclerotia in the upper 30 cm of soil decreased by 99.5-100%. This reduction can be attributed to the hot, moist conditions developed under the polyethylene sheet which are essential for the lethal effect of solarization on the pathogen.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary causes serious damage (white mould) of tomato in non-heated greenhouses in Greece. Under climatic conditions which favour the pathogen, ascospores cause infection of the crown, stem, leaves and fruits. The pathogen survives in the soil as mycelium and notably as sclerotia. It is a plurivorous and cosmopolitan parasite. Exposure of sclerotia to temperatures of 15-25°C and high soil moisture favour the formation of sexual reproduction structures, the apothecia, from sclerotia.

Chemical control of the pathogen is mainly by soil fumigants such as methyl bromide and metham sodium, which have a well-known indiscriminate biocidal action on the soil ecosystem, and broad spectrum fungicides including benzimidazoles and dicarboximides for treatment of the aerial plant parts.

Presently many researchers try to elaborate biological, physicobiological and prophylactic control measures against the disease. Antagonists such as *Coniothyrium minitans* (Huang & Hoes, 1976), *Microsphaeropsis centaureae* (Watson & Miltimore, 1975), *Gliocladium roseum* (Jones & Watson, 1969), *Trichoderma viride* (Pohjakallo & Makkonen, 1957) and *Bacillus subtilis* (Henis & Inbar, 1968) have shown effective disease control.

One physicobiological method, soil solarization with a transparent polyethylene sheet, has often been used singly or in combination with antagonists or bioregulatory substances for control of *S. sclerotiorum* in various crops (Porter & Merriman, 1985).

Our work concerns control of white mould of greenhouse tomatoes with this physicobiological method.

Materials and Methods

For two years, greenhouse plots of 25 m^2 with severe infestation with sclerotia of *S. sclerotiorum* have been used. On every plot 50 tomato plants of cv GC-204 (Carmello) were grown.

Soil was solarized for 8 weeks in summer (June-August). Soil was covered with a transparent polyethylene sheet (0.05 mm thick). Before covering, the soil was cultivated and watered. It was kept humid during the full solarization period to facilitate heat transport. Perfect flattening of the surface was necessary for good contact of soil and plastic sheet. For the same purpose the plastic was buried up to 15 cm deep into the soil at the borders of the plots.

The assessment of the efficacy of the method was based upon counts of the numbers of sclerotia present in the soil. Five samples of 1000 ml were taken at 0-10, 11-20, 21-30 and 31-40 cm deep from each plot. Representative samples for each plot and depth were obtained by aseptically mixing the five samples. Samples were taken before and after solarization. Sclerotia were separated from the soil according to the technique of Abawi & Grogan (1975), and values expressed as numbers of sclerotia per 500 ml soil. In addition, counts of infected stems and fruits were made during the vegetation period.

To calculate the efficacy of the treatments the formula of Henderson-Tilton (Püntener, 1981) was used.

Results

Following the 8 week period of solarization, the numbers of sclerotia in the control plots were reduced to 77%, 73% and 70% of the original numbers in the 0-10, 11-20 and 21-30 cm deep layers of the soil, respectively. In the solarized plots the inoculum of the pathogen decreased to 0.02, 0.00 and 0.00 sclerotia per 500 ml soil in the 0-10, 11-20 and 21-30 cm layer, respectively, which corresponds to 99.5% and 100% efficacy of the treatment (Table 1).

Table 1. Number of sclerotia of *S. sclerotiorum* in soil samples (500 ml) and % efficacy of solarization (in parentheses)

Sample	Soil layer			
	0-10 cm	11-20 cm	21-30 cm	31-40 cm
Control	5.10	1.37	0.10	0.00
Treated plots before solarization	5.46	1.35	0.08	0.00
Control after 8 weeks	3.95	1.00	0.07	0.00
Treated plots after solarization	0.02 (99.5%)	0.00 (100%)	0.00 (100%)	0.00

Infection of stems and fruits in the control plots was very high, but solarization controlled the disease with 100% efficiency (Table 2).

Table 2. Effect of solarization on infection of tomato by *S. sclerotiorum* (numbers of infected stems and fruits per 50 plants)

Treatment	Stem infections	Fruit infections
Control	12.0	53.8
Solarization	0.0	0.0

Discussion

Our results show that white mould on tomato in non-heated greenhouses can effectively be controlled by soil solarization under the summer conditions of Crete by using transparent polyethylene sheets. Similar results have been reported for other *Sclerotinia* and *Sclerotium* species: *Sclerotinia minor* (Porter & Merriman, 1983), *Sclerotium cepivorum* (El Yamani *et al.*, 1983), *Sclerotium oryzae* (Usmani & Ghaffar, 1982), *Sclerotium rolfsii* (Katan, 1981). The moist heat of the solarization treatment seems to destroy sclerotia by facilitating leakage of sugars and amino acids (Lifshitz *et al.*, 1983). Leaking makes sclerotia more vulnerable to antagonists and accelerates their decomposition.

Since solarization can also be used to control other soil-borne tomato diseases, as well as to increase soil fertility, this method can play an important role in crop protection in biological and integrated agriculture.

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BIOLOGICAL CONTROL OF OTHER SCLEROTIUM-FORMING PATHOGENS

CONTROL OF SCLEROTIAL RENEWAL OF *RHIZOCTONIA SOLANI* BY BIOLOGICAL AND CULTURAL METHODS

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Abstract

This review examines the life cycle of *Rhizoctonia solani* in relation to inoculum production and survival in a potato cropping system. Emphasis is placed on the need for a clear knowledge of the crop management system and how this can be modified and combined with biocontrol treatments such as *Verticillium biguttatum* to minimize sclerotium renewal to achieve practical control of diseases caused by *R. solani*.

Introduction

Tuber/bulb- and soil-borne sclerotia are the main inoculum of the plant pathogenic fungus *Rhizoctonia solani* in a diversity of susceptible crops such as potato, flower-bulbs and sugar beet. In potato, sclerotium formation has been studied thoroughly in relation to tuber exudates (Dijst, 1988), crop rotation (Scholte, 1992), haulm destruction (Mulder *et al.*, 1992) and biocontrol (Jager *et al.*, 1991). In this review we will concentrate mainly on biocontrol of sclerotia production in potato.

Following host stimulated germination of sclerotia, *R. solani* develops ectotrophically to produce an extensive hyphal network upon the subterranean plant in competition with co-inhabiting soil microflora. Interhyphal connections or anastomoses of *R. solani* result in hyphal networking. To overcome competition with the soil microflora, *R. solani* is able to enter a parasitic phase through production of specialized infection structures. These so-called infection cushions serve to exploit the underlying plant tissue as a protected food base to support hyphal networking and, finally, to renew sclerotia. Sclerotia are mainly formed on storage organs such as tubers, bulbs and to a lesser extent on roots or other underground plant parts. The fungus secures its long term survival by renewal of sclerotia, especially when it occurs on the host seed (tuber). Renewal of the sclerotial population is always linked with host plant senescence and the availability of host plant-derived nutrients at that time. Deprived of normal food sources, however, inocula of *R. solani* experience steady starvation over time (at least 4 years) related to the activity and nature of the associated microflora, including the mycoparasitic fungus *Verticillium biguttatum* (Velvis *et al.*, 1989). In respect to biocontrol, sclerotial inocula may be reduced either by maximizing starvation or by minimizing renewal.

In this review we aim to portray how a combination of crop management and biocontrol methods designed at minimizing sclerotium renewal are expected to have practical value for biological control of *R. solani* diseases.

Verticillium biguttatum, a mycoparasite that minimizes sclerotial renewal

The sclerotium-inhabiting fungus *V. biguttatum* is an ecologically obligate mycoparasite that specifically responds to *Rhizoctonia* species in nature. To simulate the ecological behaviour of this fungus-fungus combination *in vitro,* cellulose/nitrate agars have been employed as growth substrates and these allow hyphal networking by *R. solani* and oblige *V. biguttatum* to grow parasitically upon host mycelium (Van den Boogert & Deacon, 1994). In such a non-competitive environment on cellulosic agars, individual hyphal or sclerotial host cells are readily parasitized by *V. biguttatum* but the hyphal network as a whole is only hindered when numerous cells of the host network have been parasitized. Nevertheless, with localized infection of host hyphae only, *V. biguttatum* drastically reduces renewal of sclerotia, even in regions remote from the infection sites. This so called 'distance effect' is quantitative and depends on the number of infections within the hyphal network. Isolates belonging to *R. solani* AG3 are extremely sensitive to *V. biguttatum* resulting in complete suppression of sclerotium renewal within a 90 mm diam. colony by one single co-inoculation of the parasite at 2 cm distance from the host (Table 1). Apparently the mycoparasite is able to derive at least part of its nutrients from the host hyphal network and parasitic growth takes place at the expense of sclerotial renewal. This phenomenon is ascribed to the creation of a nutrient sink by *V. biguttatum*, consistent with biotrophy (Van den Boogert & Deacon, 1994).

Table 1. Effect of localized infection of *Verticillium biguttatum* isolates on sclerotium production by *Rhizoctonia solani* isolates (AG3), determined on co-inoculated cellulose/nitrate agar plates after 3 weeks incubation at 20°C. Data represent averages of total areas occupied by sclerotia (mm^2) within 90 mm diam. colonies in three-fold; $LSD_{5\%}$=1.2.

Isolates of *R. solani* AG3	Areas occupied by sclerotia (mm^2) per 90 mm diam colony				
	Untreated	With *V. biguttatum* isolates			
		M73	M93	M96	M99
3R-8	6.4	0.1	0.8	1.0	0.1
3R-9	21.3	0.1	3.1	2.6	0.3
3R-41	18.1	0.2	3.6	1.0	6.4

As shown in Table 1, some intraspecific variation in aggressiveness exists among isolates of *V. biguttatum* towards *R. solani* isolates as well as variation in susceptibility among host isolates belonging to the same or different anastomosis groups (see also Morris *et al.*, 1995). Other (presumptive) mycoparasites, such as the cellulolytic *Trichoderma* and *Gliocladium* spp., and *Coniothyrium minitans* which parasitize hyphal cells of *R. solani* and *Sclerotinia sclerotiorum*, respectively, on cellulosic substrates, are unable to suppress sclerotium formation at a distance. The potential to interfere with sclerotial renewal at a distance, is supposed to be a unique trait of biotrophically growing mycoparasites. If *V. biguttatum* exerts the distance effect in nature, as it does *in vitro*, then effective biocontrol would not depend on parasitism of the whole mycelium and the

attendant problem of achieving uniform distribution of the mycoparasite in soil or on plant surfaces.

Host plant debris stimulates sclerotium renewal

In nature, sclerotium production by *R. solani* is not a random process but the time and site appear to be synchronized with plant (tuber) senescence with localized preferential formation on below-ground storage organs such as tubers, turnips and bulbs. In potato, the renewal of sclerotia predominantly occurs on progeny tuber surfaces at the time of plant senescence. During senescence host plants gradually lose control of their natural defence systems and tissues become vulnerable to colonization by secondary invading phytopathogens, including *R. solani.* Although the mechanism is not fully understood, tuber exudates and volatile compounds from decaying host plants influence sclerotium formation (Dijst, 1988). Obviously, host-plant derived debris promotes sclerotium renewal at plant senescence. This stimulative effect of plant debris can be demonstrated in a mini tuber system (MTS), a system for studying *in situ* sclerotium renewal on progeny tubers (Van den Boogert *et al.*, 1994). Briefly, MTS consists of small containers filled with natural soil, artificially grown *Rhizoctonia*-inoculum and *in vitro* grown mini-tubers. Soil amendments at 0.1 to 1.0% (w/w) of fresh potato stem or stolon fragments (plant debris) stimulate sclerotium production on tuber surfaces. This stimulative effect by plant debris amendment appears to be plant-specific as debris from other sources than Solanaceae is not or only slightly stimulative. In addition, the renewal of sclerotia can be minimized by inoculating either plant debris or tuber surfaces with conidial suspensions of *V. biguttatum* at a concentration of 10^7 conidia per ml (Table 2).

Table 2. The effect of soil amendment with potato plant debris and inoculation of debris or tuber surfaces with *V. biguttatum* on production of tuber-borne sclerotia by *Rhizoctonia solani*, assayed in a soil system using *in vitro* grown minitubers (MTS)

Soil amendments (fresh stolon pieces)	Sclerotium index* (0-100)	
	Non-inoculated tubers	*Verticillium*-inoculated tubers
Control (no debris)	8.9 *a*	1.6 *c*
Debris	68.5 *b*	1.3 *c*
Verticillium-inoculated debris	0.0 *c*	0.0 *c*

Data followed by a different letter are significantly different at $P < 0.01$
* For explanation of sclerotium index see following pages

These and other experimental data support the idea that plant debris may serve as additional nutrition for *R. solani,* which enables the fungus to produce new sclerotia: interference with its additional food source or with its deposition site by inoculation with *V. biguttatum* may discourage sclerotium renewal by *R. solani.*

Haulm destruction and sclerotial renewal

In commercial potato cropping systems, tuber- and soil-borne sclerotia of *R. solani* are responsible for attack of below-ground sprouts and stolons, resulting in retarded and uneven emergence, stolon pruning, tuber malformation and sclerotium renewal on tuber surfaces. Control of tuber-borne sclerotia, known as black scurf disease, is of great economic importance for the Dutch potato growers, particularly in seed tuber production, where severe health demands apply for certified potato seed. Previously, biological control measures using *V. biguttatum* have aimed at minimizing disease by eradicating sclerotia on the seed tuber and in soil at planting, but with variable results (Jager *et al.*, 1991). The lack of predictable biocontrol following seed tuber inoculation with *V. biguttatum* is partly related to requirement of temperatures >13°C for efficient mycoparasitism. In addition, there are technical drawbacks of achieving contact between the biological agent and sclerotia, especially those dispersed throughout soil.

Haulm destruction before potato harvest both advances and eases tuber harvest. In addition, the haulm of early potato seed crops is destroyed in order to prevent aphid-mediated virus transmission or the dispersal of blight (*Phytophthora infestans*) to the tubers, and to achieve the desired tuber size. The current practice of haulm destruction requires spraying potato haulms with desiccants or herbicides at a desired moment within the growing season. Chemically-mediated haulm destruction accelerates natural plant senescence and thus black scurf disease.

Recently 'Green Crop Harvesting' (GCH) has been developed as a mechanical alternative for the current chemical method with its environmentally questionable impact (Mulder *et al.*, 1992). Analogous to conventional haulm destruction, GCH is currently performed in a still green crop at the end of the growing season just before natural plant senescence. With GCH, progeny tubers are separated from the remaining plant by cutting off the haulm at soil level and are then dug up to detach them from the root/stolon system. At this stage, while passing the digger, the tubers become accessible for targeted biocontrol. Subsequently, the tubers are laid back into the soil for a maturation period. The prevailing soil conditions at GCH are known to be conducive for tuber skin maturation but also for mycoparasitism of *R. solani* sclerotia by *V. biguttatum*. Hence, GCH provides a perfect opportunity to target biocontrol at detached progeny tubers just prior to sclerotial renewal.

Practical biocontrol of sclerotia in the field

For the past 4 years we have been working out the concept of GCH using *V. biguttatum* inoculum to control black scurf under a range of conditions in the field. The experimental design of the field plots consisted of 2 rows of 20 m long at 0.75 m distance, planted with four seed tubers per m. Each treatment was replicated four times. The potato crop was grown according to current standards for seed tuber production. At haulm destruction date (end of July), *V. biguttatum* inoculation was applied in GCH by spraying the progeny tubers with 400 l conidial suspension per ha at a concentration of 10^7 spores per ml tap water. Black scurf disease was assessed at harvest, 20 days after GCH, by visually estimating the number of sclerotia on each of 100 randomly chosen tubers. A sclerotium index was calculated as follows:

$$SI=25(1xN_1+2xN_{2-5}+3xN_{6-10}+4xN_{>10})/N_{tot},$$

in which $N_{1,2-5,6-10,>10}$ = Number of tubers with 1, 2-5, 6-10 and > 10 sclerotia per tuber; N_{tot} = total number of tubers.

In addition, the viability of randomly chosen and equally sized sclerotia collected from each of 80 tubers was determined by plating them out on water agar. After 24 h of incubation at 20°C the sclerotia were individually examined for characteristic hyphal outgrowth by *R. solani*. Apart from the tuber-borne sclerotia, stolons and root systems were also checked for viable inoculum of *R. solani*, in particular those that remained behind in the field soil after GCH. From Table 3 it is evident that GCH allows efficient biocontrol of black scurf by *V. biguttatum*. Irrespective of planting time, soil temperatures at GCH meet the optimum values for mycoparasitism by *V. biguttatum* (25°C). Those tuber-borne sclerotia that are produced in spite of inoculation suffer from mycoparasitism as shown by a decreased viability (Table 3).

Table 3. Effect of crop treatment with *Verticillium biguttatum* in GCH on amount and viability of tuber-borne sclerotia of *R. solani*, determined 20 days after haulm destruction

Location (year of experimentation)	Tuber-borne sclerotia			
	Amount (Index; 0-100)		Viable fraction (%; N=80)	
	Untreated	*Verticillium*-inoculated	Untreated	*Verticillium*-inoculated
Slootdorp (1991)	39.0	20.3 **	67	7
Rolde (1991)	29.1	10.0 **	85	7
De Krim (1991)	23.4	2.1 **	54	<
Rolde (1992)	13.5	4.9 *	37	9
Munnekezijl (1992)	51.1	35.5 **	92	26
Slootdorp (1992)	44.8	42.0 NS	84	66
Creil (1993)	8.1	0.6 **	88	<
Rolde (1994)	9.1	1.3 **	nd	nd
Munnekezijl (1994)	37.8	11.2 **	nd	nd
Creil (1994)	37.1	23.6 **	94	43

*,** Significant reduction of amount at $P < 0.05$ and $P < 0.01$, respectively (t test).
< = nil; number of sclerotia tested less than 80
nd = not determined

Although not tested in detail, it is believed that sclerotia weakened by *V. biguttatum* are susceptible to invasion by other antagonistic microorganisms which may further degrade the sclerotial tissues and thus limit their survival potential.

In GCH not only can the progeny tubers be readily targeted but other below ground plant parts may be inoculated with the mycoparasite while passing through the spray curtain on the digger. This may happen with the remains of the root and stolon systems that attend the progeny tubers while passing the GCH machinery. Although macroscopical sclerotia are certainly absent on the roots or stolons, they may harbour viable *R. solani* inoculum at high frequencies. It is known from detailed soil observations that the main survival structures of *R. solani* consist of extremely small clusters of monilioid cells associated with plant debris (Van den Boogert & Velvis, 1992). As shown in Table 4 survival of inoculum associated with plant debris can be severely affected by applications of *V. biguttatum* in GCH. Although not yet experimentally verified, it is reasonable to suggest that plant debris treatment with *V. biguttatum* may negatively affect long-term survival of *R. solani* during intercrop periods in the soil.

Table 4. Effect of crop treatment with *Verticillium biguttatum* in GCH on viability of soil-borne *R. solani* inoculum, determined on potato root residues 40 days after haulm destruction

Location (year of experimentation)	Root fragments with viable *R. solani* (%)	
	Untreated	*Verticillium*-inoculated
Rolde (1993)	19	3 **
Creil (1993)	24	1 **
Munnekezijl (1994)	69	19 **

** Significant reduction at $P < 0.01$ (t test)

Conclusions and Future Research

Most practical interest in mycoparasites centres on their potential roles in regulating the populations of plant pathogenic fungi, especially of sclerotium-forming pathogens. The biocontrol effect would be increased by targeting the mycoparasite before the renewal phase of sclerotia, such as for *R. solani* on tuber surfaces prior to plant senescence. GCH is a safe and effective method that combines an environmentally-friendly haulm destruction with targeted biocontrol under conducive conditions for tuber maturation and mycoparasitism by *V. biguttatum*.

Presently, research efforts are being concentrated on mass-production of *V. biguttatum* inoculum in solid and liquid fermentation systems aimed at acquiring a cost-effective biocontrol product that can compete with current *Rhizoctonia*-fungicides. In addition, field research should be continued on further refinement of GCH and the possibility of using other biocontrol agents to attack other pathogens that affect tuber health during maturation or storage. This would include pathogenic *Erwinia* spp., *Phytophthora infestans* and *Pythium* spp. In this respect, it is worth mentioning that *V. biguttatum* inoculum is compatible with a number of interesting antagonists active against pathogenic *Erwinia* spp., which opens perspectives for broad disease control (Van den Boogert *et al.*, 1994).

Since the beginning of rational agriculture, diseased plants or infected debris have been routinely removed by hand from the field as a general phytosanitary measure. Inoculation with *V. biguttatum* has been shown to be effective in disinfecting plant debris on a field scale. In this particular example, removal of *Rhizoctonia*-infected debris can be replaced by mechanical crop residue treatment using GCH in combination with *V. biguttatum*. Although speculative, mycoparasites and particularly those that behave as obligate mycoparasites in nature, may play an important role in preventing sclerotium renewal on plant debris. For practical exploitation, targeting the time of application of inoculum is conditional for successful biocontrol. GCH and the potato-*R. solani* pathosystem is a good example. Future research will be directed on other pathosystems with *R. solani* in which *V. biguttatum* or other mycoparasites can be applied to plant debris in order to develop new phytosanitary measures that prevent survival of sclerotium-forming pathogens.

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BIOCONTROL OF DIFFERENT *RHIZOCTONIA SOLANI* ANASTOMOSIS GROUPS WITH THE MYCOPARASITE *VERTICILLIUM BIGUTTATUM*

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Abstract

Verticillium biguttatum was isolated from *Rhizoctonia solani* sclerotia from UK potato tubers and identified using morphological and physiological characteristics. Two UK isolates and a Dutch isolate were then compared for activity against *R. solani* in *in vitro* assays and *in vivo* biocontrol assays. *In vitro* assays showed that a range of *R. solani* anastomosis groups (AGs) were colonised by *V. biguttatum* from both conidial and mycelial inocula. *V. biguttatum* produced non-volatile metabolites on potato dextrose agar (PDA) that inhibited mycelial extension of these *R. solani* AGs. Two different hydroxymethyl-phenols were isolated from ethyl acetate extracts of 24-day-old filtrates of static *V. biguttatum* cultures in modified Czapek Dox liquid medium and each inhibited *R. solani* AG3 at 138 $\mu g\ ml^{-1}$ in PDA. During an *in vivo* biocontrol assay, barley stunt disease (*R. solani* AG8) was reduced by up to 73% in trays after application of 21-day-old maizemeal-perlite inoculum of *V. biguttatum* at 1% (w/w) to potting mixture artificially infested with *R. solani*. However, lettuce bottom rot (*R. solani* AG1, AG2-1 and AG4) and *R. solani* soil propagule density were not reduced after application of *V. biguttatum* inoculum at 1 kg m^{-2} soil surface in a biocontrol assay in a polyethylene tunnel. Here, there was no biocontrol effect even though *V. biguttatum* propagule densities reached 6.5×10^6 c.f.u.s g^{-1} soil. There was no evidence of production of plant growth stimulators by the mycoparasite in either *in vivo* assay. This study showed *V. biguttatum* is indigenous to the UK, can effect mycoparasitism and antibiosis against different *R. solani* AGs and has potential for biocontrol of diseases caused by *R. solani* AGs other than AG3.

Introduction

The mycoparasite *Verticillium biguttatum* was first isolated in The Netherlands from sclerotia of *Rhizoctonia solani* AG3 on black scurf-infected potato tubers (Jager *et al.*, 1979). *V. biguttatum* may also be associated with a decline in the severity of black scurf after nematicide application in the UK (Hide & Read, 1991). When used in conjunction with 20% of the recommended dosage of the fungicide pencycuron, *V. biguttatum* can give effective control of black scurf (Jager & Velvis, 1986).

In vitro antagonism assays have shown *R. solani* AGs 1-4, some binucleate *Rhizoctonia* and *Ceratobasidium* species and *Sclerotinia sclerotiorum* to be susceptible to mycoparasitism by *V. biguttatum* (Van den Boogert *et al.*, 1989). Also, there is evidence for obligate mycoparasitism of *R. solani* AG3 on potato roots and stolons in soil by *V. biguttatum* (Van den Boogert, 1989). No effective *in vitro* inhibition of *R. solani* by metabolites of *V. biguttatum* has been reported in the literature (Van den Boogert & Jager, 1984).

Consequently, mycoparasitism of *R. solani* AG3 on potato tubers, stolons and roots is thought to be the major mechanism of action in the suppression of black scurf by *V. biguttatum* (Van den Boogert, 1989; Van den Boogert *et al.*, 1989; Van den Boogert & Deacon, 1994).

In the study described we initially isolated *V. biguttatum* from the UK and then examined mechanisms of action which may be involved in biocontrol of different *R. solani* AGs by *V. biguttatum*. Finally, we assessed the potential *in vivo* for biocontrol of two plant diseases caused by *R. solani* AGs other than AG3.

Isolation from the UK

Although a world-wide distribution of *V. biguttatum* in association with *R. solani* on potato tubers has been demonstrated, the mycoparasite has not previously been isolated in the UK (Van den Boogert & Saat, 1991). So, as the initial step in our study, isolates of *V. biguttatum* were sought from sclerotia of *R. solani* from UK potato tubers (Morris *et al.*, 1992).

We obtained three *V. biguttatum* isolates from 39 samples of infected tubers so the mycoparasite is indigenous to the UK but appears uncommon. However, fungicide treatment of tubers and desiccation of *R. soalni* sclerotia during transportation can greatly affect the frequency of *V. biguttatum* isolation (Jager & Velvis, 1986). So, the mycoparasite may be more common in the UK than was apparent. Techniques for direct isolation of *V. biguttatum* from soil (Morris *et al.*, 1995) may increase the isolation frequency.

In vitro antagonism assays

The ability of two UK and one Dutch isolate of *V. biguttatum* to effect mycoparasitism and antibiosis against *R. solani* AG1, AG2-1, AG3, AG4 and AG8 in a range of *in vitro* anatagonism assays was assessed (Morris, 1993).

In dual culture on PDA, all the *R. solani* AGs were overgrown by the *V. biguttatum* isolates and on distilled water agar, mycoparasitic colonisation was confirmed using light microscopy. There were quantitative differences in colonisation of PDA precolonised with different *R. solani* AGs when conidial inoculum of *V. biguttatum* was used in comparison to mycelial inoculum. Also, ageing of *R. solani* reduced colonisation by *V. biguttatum* from conidial inoculum but not from mycelial inoculum. Further, the processes of colonisation and overgrowth were affected differently by the range of AGs. Nevertheless, each *R. solani* AG assayed was susceptible to mycoparasitism by *V. biguttatum*.

A cellophane agar overlay assay on PDA showed antibiosis by non-volatile metabolites of the three *V. biguttatum* isolates against all the *R. solani* AGs. Subsequently, thin layer chromatographic (TLC) bioautography showed these isolates produced two heat stable antifungal metabolites in static liquid culture in a modified Czapek-Dox medium. After purification by preparative TLC and identification by spectroscopic analysis the two metabolites were found to be different hydroxymethyl-phenols. Each metabolite inhibited mycelial extension of *R. solani* AG3 at 138 $\mu g\ ml^{-1}$ in PDA. Thus, *V. biguttatum* has the physiological capability to effect antibiosis against *R. solani* and so this mechanism may play a role during biocontrol.

In vivo biocontrol assays

In vivo mechanisms of action and the biocontrol potential of *V. biguttatum* using a barley stunt disease (*R. solani* AG8) seedling model system (Morris *et al.*, 1993) and the lettuce bottom rot disease complex (*R. solani* AG1, AG2-1 and AG4) were then examined.

Barley stunt disease was reduced by up to 73% in seedlings after application of 21 day perlite-maizemeal inoculum of *V. biguttatum* at 1% (w/w, $1x10^5$ c.f.u.s g^{-1}) to potting mixture that had been artificially infested with approximately 24.0 c.f.u.s g^{-1} of *R. solani* AG8. Biocontrol was increased by approximately 10% if *R. solani* and *V. biguttatum* inoculated trays were incubated for only 1 day prior to planting barley and biocontrol increased progressively throughout each 8 day experiment. If barley was planted after inoculated trays had been incubated for 7 days there was no disease at all. Thus, *V. biguttatum* appeared to affect *R. solani* both before and after the initiation of disease. This is consistent with mycoparasitism being the major mechanism of action of *V. biguttatum* during biocontrol of *R. solani* (Van den Boogert, 1989; Van den Boogert *et al.*, 1989). Conversely, in polyethylene tunnel experiments lettuce bottom rot and *R. solani* soil propagule density were not reduced after application of *V. biguttatum* inoculum at 1 kg m^{-2} soil surface. Here, there was no biocontrol effect even though *V. biguttatum* propagule densities reached 6.5 x 10^6 c.f.u.s g^{-1} soil and only declined slowly during each experiment. There was no evidence of production of plant growth stimulators or phytotoxic metabolites by the mycoparasite in either *in vivo* biocontrol assay method as plant weight was not affected by application of *V. biguttatum* inoculum.

Thus, our *in vivo* studies do not contradict the theory that mycoparasitism is the major mechanism of action (Van den Boogert, 1989; Van den Boogert *et al.*, 1989; Van den Boogert & Deacon, 1994) and showed that plant growth stimulation is not a mechanism of action of *V. biguttatum*. Encouragingly, there was evidence of potential for biocontrol of barley stunt disease caused by *R. solani* AG8.

Conclusions

1) *V. biguttatum* is indigenous to, but uncommon in, the UK.

2) *V. biguttatum* can effect both mycoparasitism and antibiosis against *R. solani* AG1, AG2-1, AG3, AG4 and AG8 *in vitro*.

3) *V. biguttatum* has potential as a biocontrol agent of plant diseases other than those caused by *R. solani* AG3 on potato but the target disease needs to be chosen carefully.

Acknowledgement

This work was funded by the Biotechnology and Biological Sciences Research Council and the Ministry of Agriculture, Fisheries and Food for England and Wales.

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THE EFFECT OF SOIL FUNGI ON *RHIZOCTONIA CEREALIS* V.D. HOEVEN AND *RHIZOCTONIA SOLANI* KÜHN

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Abstract

In Poland, in 1993 and 1994 sharp eyespot of winter wheat was present at the highest level before harvest and amounted to 2.7-4.7% and 4.7-20.7%, respectively. The greatest infection occurred in fields where the preceding crops had been winter rape and sugar beet. In 1993, populations of soil fungi were isolated from the fields of winter wheat where four different preceding crops had been cultivated. In agar plate tests, microbial communities from the field in which cereals (wheat, maize) had been cultivated as preceding crops were found to be the least inhibitory towards *Rhizoctonia cerealis* and *Rhizoctonia solani*. The influence of five species of soil fungi which had a restrictive biotic effect on the two pathogens was investigated in a greenhouse experiment with spring wheat. It was found that *Fusarium culmorum* in the presence of *R. cerealis* reduced the occurrence of sharp eyespot while *Actinomyces* spp. and *Penicillium griseofulvum* had the same effect on *R. solani*. The mixture of all the saprophytic species investigated also had a suppressive influence in relation to both pathogens.

Introduction

Fungi of the genus *Rhizoctonia* are commonly found on wheat in various regions where this crop is cultivated and disease aetiology has been widely examined (Weller *et al.*, 1986; Pumphrey *et al.*, 1987; Smiley *et al.*, 1990). Some soils suppressive to the development of *Rhizoctonia* species are known and recently, Lucas *et al.* (1993) discovered that a previous crop can influence this suppressiveness. In order to find the source of that suppressive activity, fungal populations have been isolated from soil in which different previous crops had been grown and the effect of selected species antagonistic to *R. cerealis* and *R. solani* investigated *in vitro* using potato dextrose agar (PDA), and *in vivo*, in soil in pot experiments (Henis *et al.*, 1978; Lin & Baker, 1980; Chet & Baker, 1980).

This paper presents observations of the changes in occurrence of diseases caused by *R. solani* and *R. cerealis* on winter wheat in relation to the previous crops cultivated.

Field investigations (Manieczki, 1993)

Three observations on the occurrence of foot rot diseases of winter wheat were made during 1993. The experiments were carried out in Manieczki in adjacent fields of wheat cultivated after four different previous crops: wheat, maize, winter rape and sugar beet. The objective of the investigations was to determine the effect of the preceding crop on the level of sharp eyespot caused by species of *Rhizoctonia* (*R. cerealis* and *R. solani*). In the first trial, 300 plants (6 x 50) from each field were evaluated, whereas in the second and third trial, 150

plants (6 x 25) were examined. During the first observation (March 17, 2-3 leaf stage), sharp eyespot symptoms were present on only a single plant. At the beginning of anthesis (19 April), there was a significant increase in the number of plants infected in the field where sugar beet was the previous crop compared to fields where wheat and rape had been grown previously. At milky ripe (24 June) the level of stem infection was similar in all fields regardless of previous crops (Table 1). Symptoms of foot rot diseases were also most prevalent at milky ripe.

Table 1. The occurrence of sharp eyespot in winter wheat in relation to the preceding crop (Manieczki, 1993)

Preceding crop	Percentage of infected plants*		
	17 March	19 April	24 June
Wheat	0 *a*	0 *a*	2.7 *abc*
Maize	1 *abc*	1.3 *ab*	3.3 *abc*
Rape	0 *a*	0 *a*	4.7 *c*
Sugar beet	0 *a*	5.3 *bc*	3.3 *bc*

* Means followed by the same letter do not differ significantly

Isolations of soil fungi from fields of winter wheat (Manieczki, 1993)

In May 1993, isolations were made from soil taken from the fields of winter wheat cultivated after four different previous crops. The isolations were carried out by Warcup's method modified by Manka (Manka, 1974). The four populations of soil fungi obtained in this way were subject to qualitative and quantitative analysis (Table 2). The dominant fungi in these populations belonged to the genera: *Penicillium*, *Fusarium*, *Trichoderma* as well as the family Dematiaceae. To determine the biotic effect of these isolates on *R. cerealis* and *R. solani*, agar plate dual culture tests were carried out (Manka, 1973). A multinuclear strain of *R. solani* used in the experiments was isolated from wheat while a bi-nuclear strain of *R. cerealis* was obtained from the collection of the Academy of Agriculture in Poznan. In addition to the individual biotic effect assayed by the plate method, the total biotic effect of the whole populations of the soil fungi isolated from the fields on *R. cerealis* and *R. solani* was also determined (Manka, 1973) (Table 3). The restrictive (positive) effect on *R. solani* was only found in the fungal population coming from the field where rape had been grown as previous crop.

The populations from the fields in which maize or wheat had been previously cultivated promoted the growth of both pathogens. In most cases, the total biotic effect proved to be less conducive to the growth of *R. solani* than *R. cerealis*. The analysis of the interaction between the isolates of the soil fungi and the pathogens by the plate method made it possible to identify five isolates which had the greatest restrictive effect on both *R. cerealis* and *R. solani*. The restrictive effect on both pathogens was investigated in a greenhouse experiment.

Table 2. Populations of soil fungi from fields of winter wheat in which different preceding crops had been cultivated

Preceding crop	Number of isolates of dominant genera		Total number of isolates
Wheat	*Penicillium* spp.	70	
	Family Dematiaceae	31	
	Fusarium spp.	7	133
	Trichoderma spp.	3	
Maize	*Penicillium* spp.	89	
	Family Dematiaceae	37	
	Fusarium spp.	8	184
	Trichoderma spp.	15	
Rape	*Penicillium* spp.	48	
	Family Dematiaceae	33	
	Fusarium spp.	10	121
	Trichoderma spp.	6	
Beet	*Penicillium* spp.	51	
	Family Dematiaceae	26	107
	Fusarium spp.	3	
	Trichoderma spp.	1	

Table 3. Total biotic effect of the populations of fungi from soil in which different preceding crops had been cultivated

Preceding crop	Total biotic effect of the population of soil fungi on:	
	Rhizoctonia solani	*Rhizoctonia cerealis*
Wheat	– 198	– 187
Maize	– 164	– 362
Rape	+ 16.5	– 46
Beet	– 14	– 135

Greenhouse experiment

The isolates of soil fungi antagonistic to *R. cerealis* and *R. solani* which were selected on the basis of the biotic test were subsequently used in a pot experiment using spring wheat grown in a greenhouse. These were: *Penicillium griseofulvum* (B 16), *Fusarium culmorum* (R 11), *Actinomyces* sp. (R 23), *Trichoderma hamatum* (= *T. strictipilis* - K 204), *Trichoderma viride* (B 30).

The fungi used in this experiment were grown under sterile conditions in potting medium enriched with 5% maize flour. Portions of this inoculum were added to the soil in pots (25 ml per pot) and fifteen seeds of spring wheat (cv. Sigma) were sown in each pot. The following controls were used: absolute control - without fungi; relative control - with single isolates of pathogens and soil fungi. The restrictive effect of soil fungi on each of the

pathogens was investigated by bi-fungal combinations. Additional combinations were provided by pots in which the individual pathogens were mixed with all isolates of soil fungi. Two weeks after the start of the experiment, the number of germinated plants and the height of the plants in experimental combinations were determined (Table 4).

Table 4. Effect of introduction of pathogens, soil fungi and their combinations to steamed soil on the growth of wheat

Combinations**	Number of germinating plants	Height of plants*
Control 0	88	24.5 *f*
RC	89	21.6 *cdef*
RS	89	21.9 *cdef*
R 11	90	24.6 *f*
B 30	89	23.4 *ef*
K 204	89	24.2 *f*
R 23	88	23.9 *ef*
B 16	89	23.0 *def*
RC + R 11	90	19.5 *abc*
RC + B 30	86	19.6 *abc*
RC + K 204	87	20.0 *bc*
RC + R 23	90	20.9 *cde*
RC + B 16	88	17.7 *ab*
RS + R 11	86	21.7 *cdef*
RS + B 30	90	23.2 *ef*
RS + K 204	89	17.2 *a*
RS + R 23	88	20.2 *bcd*
RS + B 16	89	21.1 *cde*
RC + other soil fungi	88	19.5 *abc*
RS + other soil fungi	84	20.0 *bc*

* Means followed by the same letter do not differ significantly
** See text for full names of fungi

It was found that the presence of pathogenic or saprophytic fungi had no effect on plant growth. *R. cerealis* in combination with saprophytic fungi resulted in a significant decrease in plant growth compared to the absolute control. A similar effect was observed in the case of *R. solani* coupled with R 23, B 16 and K 204. The addition of isolate B 16 to *R. cerealis* resulted in restricted plant growth in comparison with the pathogen only control. Similarly, isolate K 204 in combination with *R. solani* caused a decrease in growth in comparison with the control groups containing RC and RS.

At the termination of the experiment, the occurrence of the symptoms of sharp eyespot was analysed by determining the percentage of infected plants and the degree of infection on a scale of 0-3 (Table 5).

Table 5. Effect of introduction of pathogens, soil fungi and their combinations to steamed soil on sharp eyespot in wheat

Combinations**	Percentage of infected plants*	Average degree of infection*
Control 0	0 *a*	0 *a*
RC	81.9 *g*	2.5 *de*
RS	33.0 *cde*	2.4 *de*
R 11	0 *a*	0 *a*
B 30	0 *a*	0 *a*
K 204	0 *a*	0 *a*
R 23	0 *a*	0 *a*
B 16	0 *a*	0 *a*
RC + R 11	35.7 *de*	2.1 *cde*
RC + B 30	12.7 *b*	1.5 *bcd*
RC + K 204	54.3 *ef*	2.7 *e*
RC + R 23	66.3 *fg*	2.6 *e*
RC + B 16	25.2 *bcd*	2.4 *de*
RS + R 11	17.6 *bc*	1.0 *b*
RS + B 30	36.0 *de*	2.4 *de*
RS + K 204	25.9 *bcd*	2.8 *e*
RS + R 23	12.4 *b*	2.5 *de*
RS + B 16	11.9 *b*	1.3 *bc*
RC + other soil fungi	16.7 *bc*	2.6 *de*
RS + other soil fungi	0 *a*	0 *a*

* Means followed by the same letter do not differ significantly
** See text for full names of fungi

The presence of fungal isolates R 11, B 30, K 204 and B 16 as well as a mixture of all isolates resulted in a decrease in the number of wheat plants infected with *R. cerealis*. The presence of fungal isolates R 23 and B 16 as well as a mixture of all isolates resulted in a decrease in the number of wheat plants infected with *R. solani*. Saprophytic fungi did not result in a decrease in the degree of infection with *R. cerealis*. A decrease in the degree of infection by *R. solani* was by the isolates R 11, B 16 and the mixture of all isolates.

Field investigations (Zlotniki, 1994)

In 1994 the occurrence of sharp eyespot and other foot rot diseases was observed in two fields of winter wheat in Zlotniki near Poznan in which barley and winter rape had been cultivated as previous crops. The observations were repeated 3 times during the vegetative season. Each time 150 (6 x 25) plants in each field were analysed. In the first observation (21 April) (tillering), only single plants showed symptoms of *Fusarium* stem and root rot.

The occurrence of sharp eyespot was discovered on 14 June (milky ripe) and on 27 July (waxy ripe). The percentage of the infected plants as well as the degree of infection in a 0-3 scale were determined (Table 6). The plant infection was relatively low and no differences between times of observations and previous crops were found.

Table 6. The occurrence of sharp eyespot in relation to the preceding crop (Zlotniki, 1994)

Preceding crop	14 June		27 July	
	Percentage of infected plants*	Average degree of infection*	Percentage of infected plants*	Average degree of infection*
Barley	2.0 *a*	0.7 *a*	5.3 *a*	1.3 *a*
Rape	6.0 *a*	1.3 *a*	3.3 *a*	0.7 *a*

* Means followed by the same letter do not differ significantly

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BIOLOGICAL CONTROL OF *BOTRYTIS CINEREA* OF GRAPEVINE: CRITICAL ANALYSIS OF THE RESULTS

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Abstract

The results obtained in a 12 year period by using *Trichoderma* spp. as a biocontrol agent against *Botrytis cinerea* on grape are critically reviewed. Used alone, *Trichoderma* spp. achieved a disease control similar to that obtained by using traditional fungicides, such as dichlofluanid and chlorothalonil. However, a certain degree of variability was observed in the different years/trials. Integrated control strategies, based on the application of *Trichoderma* spp. and one dicarboximide spray at changing of colour (C) achieved more consistent results.

Introduction

Control of grey mould of grapevine, incited by *Botrytis cinerea* Pers., has been complicated by the development of strains of the pathogen resistant to benzimidazole and/or dicarboximide fungicides. This phenomenon is quite widespread in all grape-growing areas (Gullino & Garibaldi, 1986). Moreover, as old chemicals are withdrawn from the market, because of toxicological problems or lack of interest for their re-registration, and fewer new ones are available (Gullino, 1992), the need for alternative control measures that help avoid exclusive use of fungicides is becoming more important. Biological control is one such measure.

Trichoderma spp. have been widely investigated as possible biocontrol agents. Most attempts at using *Trichoderma* on grape have been carried out in Europe, particularly in France (Dubos, 1992) and Italy (Bisiach *et al.*, 1985; Gullino, 1992). More recently, *T. harzianum* was applied as a biocontrol agent on table grape in Israel (Elad & Zimand, 1991).

A critical analysis of the results obtained in Italy in 12 years of trials carried out using *Trichoderma* spp. is presented here.

Materials and Methods

From a number of strains of *Trichoderma* derived from a screen carried out on hundreds of microorganisms, four *Trichoderma* strains were selected for further study. Selection involved consideration of potential beneficial traits such as satisfactory biocontrol activity as well as potential problems such as production of toxic metabolites (Altomare *et al.*, 1990).

Treatments with *Trichoderma* were generally carried out according to the "standard" method, based on application at phenological stages: end of flowering (A), touching of berries (B), changing of colour (C), 21 days before harvest (D). In some cases, one extra treatment was applied at full flowering (A'). *Trichoderma* was applied directly on the bunches as a suspension of at least 10^7 conidia ml^{-1}.

Trials under field conditions were mostly carried out on varieties such as "Moscato" and "Barbera", which are very susceptible to the disease.

Results

Among the different microorganisms tested, several isolates of *Trichoderma* spp. proved to be the most effective and reliable biocontrol agents. The protection offered by such biocontrol agents was often similar to that offered by traditional, contact fungicides, such as dichlofluanid and chlorothalonil, but lower than that obtained by using compounds specifically active against *B. cinerea*, provided there was no pathogen resistance to fungicides.

Table 1. Efficacy of *Trichoderma* spp. against grey mould of grape in trials carried out during 1981-1993

Year	Variety	% efficacy	Year	Variety	% efficacy
1981	Grignolino	43 (34) *	1989	Moscato	28 (20)
				Moscato	56 (28)
1983	Barbera	62 (11)			
			1990	Moscato	20 (4)
1984	Barbera	48 (25)		Moscato	40 (4)
1985	Moscato	52 (17)	1991	Moscato	67 (8)
1986	Moscato	33 (19)	1992	Moscato	58 (18)
	Barbera	20 (11)		Moscato	0 (20)
				Moscato	56 (14)
1987	Moscato	25 (38)			
			1993	Moscato	20 (12)
1988	Moscato	65 (8)		Moscato	38 (4)
	Moscato	75 (13)			
Average efficacy		42 (16)			

* The percentage of infected berries in the control plots in each trial is given in parentheses

The average efficacy shown by *Trichoderma* was 42% (Table 1). However, a significant degree of variability in the biocontrol activity was observed in the different trials and/or years. Such variability can be, at least partially, explained by the poor survival of *Trichoderma* spp. in the grape phylloplane, observed during the warmest period of the season.

At least three treatments with antagonists are necessary in order to achieve a satisfactory level of disease control; the treatment placed at the end of flowering (A) appears to be particularly critical.

The selection of mutants of *Trichoderma* resistant to benzimidazoles and dicarboximides (Gullino *et al.*, 1986) permitted the design of integrated control strategies based on alternation of one chemical treatment with 2-3 applications of the antagonist (Gullino *et al.*, 1991). The best timing for the chemical treatment in Northern Italy is at changing of colour of berries (C). However, in Northern Italy, where growers spray "Moscato", the most susceptible variety to *B. cinerea* infections, only 2-3 times/season, and the less susceptible varieties 1-2 times/season even when conditions are very favourable to grey mould infections, the use of *Trichoderma* in alternation with one chemical spray does not always lead to a significant reduction in fungicide use. Reduction in fungicide use might be more significant in cultural situations where the number of sprays/season is higher.

Acknowledgement

Research supported by a grant from Ministero delle risorse agrigole e forestali (Progetto Finalizzato Lotta biologica e integrata. Sottoprogetto: Viticoltura).

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BIOLOGICAL CONTROL OF *BOTRYTIS* SPP. BY SUPPRESSION OF SPORULATION ON NECROTIC TISSUE

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Abstract

Biological control of *Botrytis* spp. may involve the use of antagonists to prevent infection of healthy tissue or reduce pathogen sporulation and the survival of resting propagules such as sclerotia. Biocontrol based on interactions between saprophytic fungi and *Botrytis* spp. in necrotic plant tissue may affect the sporulation capacity of the pathogen resulting in a slower build-up of an epidemic in the field. The advantage of such an approach is the long interaction time between the saprophytically growing mycelium of the antagonist and of the pathogen compared to antagonism on healthy leaves, where the pathogen may escape by rapid penetration of the leaf.

Antagonists were screened under laboratory conditions for ability to suppress sporulation of *Botrytis* spp. on dead leaf tissue. Under continuous moist conditions, *Alternaria* spp., *Gliocladium* spp., *Chaetomium globosum*, *Trichoderma* spp. and *Ulocladium* spp. inhibited sporulation of *Botrytis* spp. completely. In bioassays with interrupted wetness periods, *Alternaria* spp., *C. globosum*, *U. atrum* and *Aureobasidium pullulans* suppressed sporulation of *Botrytis* almost completely, but *Trichoderma* spp. and *Gliocladium* spp. were much less effective.

The ability of the antagonists *A. pullulans*, *C. globosum*, *G. catenulatum* and *U. atrum* to colonize dead lily leaf tissue and to protect the substrate from colonization by naturally occurring airborne inoculum of *Botrytis* spp. was tested under field conditions. The performance of antagonists was quantified by determining the sporulation potential of *Botrytis* spp. after incubation of the necrotic leaves in moist chambers after five to six days of exposure to field conditions. *U. atrum* consistently reduced the leaf area covered with conidiophores of *B. cinerea* by 80-96% compared to the control in a sequence of nine experiments performed under various microclimatic conditions. *C. globosum* was effective in three out of nine experiments. The antagonists *A. pullulans* and *G. catenulatum* and the fungicide Daconil M (chlorothalonil/maneb) had no inhibitory effect on sporulation of *B. cinerea*.

Introduction

Botrytis spp. cause devastating diseases in a great many crops, both dicots and monocots, and biological control is being investigated as an alternative control strategy to the routine use of chemicals. However, prevention of infection of healthy tissue by application of an antagonist to compete with the pathogen at the infection site is not a very promising approach, due to the short interaction period and the pathogen's capacity to infect quickly

under favourable conditions, thus escaping from the influence of the antagonist (Fokkema, 1993). A second approach with sclerotia-forming pathogens is reduction of survival of sclerotia through the use of antagonists. Since survival of sclerotia is not an important factor in the epidemiology of *Botrytis* spp., we focused on a third link in the disease cycle where *Botrytis* colonizes necrotic tissue and sporulates abundantly. For effective control here an antagonist should be able to colonize necrotic plant tissue, survive under field conditions, and compete with *Botrytis* spp. for the utilization of nutrients so as to reduce the sporulation of the pathogen. This should result in a decrease in the build-up of an epidemic (Sutton & Peng, 1993; Köhl *et al.*, 1995b).

Materials and Methods

In a field experiment, plots of onions were sprayed with a spore suspension of *Gliocladium roseum* as a potential antagonist of *Botrytis squamosa* and/or *B. cinerea*. In comparison, the effect of colonisation of necrotic tissue by a saprophyte on sporulation of *Botrytis* spp. was simulated by removing leaves with ≥ 50% necrotic area each week. The *Botrytis* spore load of the air was assessed by trapping spores with Rotorods.

Several saprophytes isolated from dead onion leaves and other sources were screened in a bioassay on sterilised onion leaf pieces for their capacity to reduce sporulation of *B. cinerea* and *B. aclada* inoculated 8 and 24 hrs, respectively, before the antagonist. By interrupting the humid conditions with a 9 hr dry period on the first day of incubation only, or on three consecutive days, the effect of such dry periods on the relative success of the saprophytes to suppress *Botrytis* spp. was assessed by scoring the number of conidiophores per unit leaf area.

Using the most promising isolates from the laboratory test, nine field experiments were performed. Daconil M (chlorothalonil/maneb) and water served as controls. The antagonists were sprayed on sterilised necrotic lily leaves mounted in groups of ten on a wire and incubated for 5-6 days in a sugar beet field at leaf canopy height. After exposure in the field, the leaf pieces were collected, incubated in moist chambers for 3 days, and the percentage of the tissue bearing sporulating *Botrytis* was assessed. In another experimental set-up spore germination of the antagonists was assessed after 18 hours of field incubation. Microclimatic data were collected throughout all the experiments.

Results and Discussion

Above the plots with onions sprayed with *G. roseum*, the *Botrytis* spore load of the air, mostly *B. cinerea*, did not differ from the untreated control. Removal of necrotic leaves, however, reduced the number of conidia to about half the concentration of the control, from about 500 conidia per m^3 air to about 200. These data prove the potential for reduction of *Botrytis* inoculum by antagonists which prevent the pathogen from exploiting necrotic tissue. It also indicates that spore loads in the air over a field, even for a ubiquitous fungus like *B. cinerea*, are mostly from local origin.

Under laboratory conditions with continuous high moisture levels, a great variety of saprophytes, such as *Alternaria* spp., *Gliocladium* spp., *Chaetomium globosum*,

Trichoderma spp. and *Ulocladium* spp. completely suppressed sporulation of *B. cinerea* on dead onion leaf tissue. The suppressive effect of *Trichoderma* spp. and *Gliocladium* spp., however, disappeared when the moist periods were interrupted by even only one dry period. *Alternaria* spp., *C. globosum*, *U. atrum* and *Aureobasidium pullulans* remained highly effective under conditions of interrupted moist periods. Under such conditions the sporulation of *B. cinerea* on the necrotic tissue was also delayed to some extent.

Screening *A. pullulans*, *C. globosum*, *G. catenulatum* and *U. atrum* on dead lily leaves in the field yielded variable results. The fungicide Daconil M proved ineffective, and so did *A. pullulans* and *G. catenulatum*. *C. globosum* was effective in some of the experiments. Only *U. atrum* yielded a consistent reduction of sporulation of *B. cinerea* to nearly zero (Table 1; Köhl *et al.*, 1995a; 1995b). The mixture of *C. globosum* with *U. atrum* generally was as effective as *U. atrum*, but in a single case it had a reduced effect, being equal to that of *C. globosum*.

Table 1. Effect of treatment of necrotic lily leaves with various saprophytes and a fungicide on sporulation of *B. cinerea* on the treated leaves after field exposure followed by moist laboratory incubation. (Results of 5 representative experiments out of 9.)

Treatment	Experiment no.				
	1	2	3	4	5
Water	15[1)] *a*	24 *b*	19 *a*	30 *a*	23 *a*
Daconil M	16 *a*	31 *ab*	13 *a*	26 *ab*	16 *ab*
A. pullulans	25 *a*	32 *ab*	14 *a*	26 *ab*	17 *ab*
C. globosum	5 *bc*	31 *ab*	15 *a*	34 *a*	10 *b*
G. catenulatum	14 *ab*	45 *a*	17 *a*	15 *bc*	24 *a*
U. atrum	2 *c*	1 *c*	1 *b*	3 *d*	2 *c*
C. globosum/*U. atrum* in mixture	6 *bc*	2 *c*	17 *a*	6 *cd*	2 *c*

1) Percentage leaf area covered with conidiophores of *Botrytis cinerea*. Values followed by different letters differ significantly ($P < 0.05$).

Germination of the spores of the saprophytes was strongly influenced by environmental conditions. *C. globosum* was more sensitive to unfavourable conditions than *U. atrum*. More than 10 hrs of leaf wetness in combination with a temperature of $\geq 10^{\circ}C$ was necessary for nearly complete spore germination (Table 2).

Table 2. Effect of moisture and temperature on germination of conidia of *Chaetomium globosum* and *Ulocladium atrum* on dead lily leaves incubated under field conditions for 18 hours

Experiment no.	Germination rate (%)		Duration of leaf wetness (hrs)	
	C. globosum	*U. atrum*	Total	>10°C
1	70	95	18.0	18.0
2	0	0	0.0	0.0
3	27	71	15.0	11.5
4	0	11	18.0	8.5
5	0	<1	8.5	8.5
6	0	2	9.0	3.5
7	87	99	18.0	18.0
8	2	16	4.0	4.0

The experiments show that *U. atrum* is the only one of the saprophytes examined which has the intrinsic properties to withstand the harsh environmental conditions to which biocontrol agents will be exposed in the field. It has shown good results in suppression of sporulation of *B. cinerea* on dead tissue. Further experiments to test its effectiveness as a biocontrol agent for the protection of various crops are presently being performed.

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BIOLOGICAL CONTROL OF *BOTRYTIS CINEREA* IN GREENHOUSE CROPS

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Abstract

For the last six years we have been working on biological control of *Botrytis cinerea* causing grey mould on tomato plants. The work concentrated on the use of compost extracts and developed into studies with fungal and bacterial antagonists. Compost extracts were highly effective against *B. cinerea* on detached bean leaves in the laboratory and moderately effective against *B. cinerea* on young bean plants in the laboratory or on tomatoes in the greenhouse. Fungal antagonists were highly effective against *B. cinerea* on detached bean leaves and young bean plants. Timing of application of antagonists and inoculation with *B. cinerea* as well as the temperature of the incubation influenced the level of control achieved. In the greenhouse, only strains of *Cladosporium* spp. were found to be very effective in controlling grey mould. The effectiveness of bacterial antagonists, selected from the phylloplane of greenhouse vegetables as well as from compost extracts, ranged from 0 to 90% on detached bean leaves.

Introduction

Grey mould caused by *Botrytis cinerea* is one of the most destructive diseases of greenhouse crops world-wide. Its control by fungicide application is becoming increasingly difficult due to the predominance of strains resistant to the most effective fungicides (Gullino, 1992). Environmental control of grey mould by use of energetically expensive systems, like automated ventilation to reduce humidity, is not always possible due to high costs and cannot be done in plastic greenhouses. As an alternative, the introduction of biocontrol systems using filamentous fungi, yeasts and bacterial antagonists as well as compost extracts has been studied for several years (Edwards & Seddon, 1992; Elad *et al.*, 1992; Malathrakis & Klironomou, 1992; Marois, 1992; McQuilken *et al.*, 1993; Edwards *et al.*, 1994). For the last six years we have been investigating the biocontrol of grey mould on several plants in the laboratory and on tomatoes grown in plastic greenhouses using fungal and bacterial antagonists as well as watery compost extracts. This paper summarises this work.

Biocontrol by compost extracts

The composts used were made from straw, cow manure, sheep manure, chicken manure and chicken manure + seaweed, by the standard procedure of composting. Extracts of these composts, filtered through cheesecloth, were initially tested in the laboratory and afterwards in the greenhouse.

To prepare extracts for laboratory experiments each compost, either alone or amended with dextrose (1%), or peptone (0.5%), or dextrose (1%) + peptone (0.5%), was mixed with water 1/8 (w/v) and placed in 500 ml Erlenmeyer flasks on an orbiting incubator at 22°C for 2 days, at 200 rpm. For greenhouse experiments, compost mixed with water 1/8 (w/v) was fermented in 20 l barrels at ambient temperature (10-15°C) for 8 days with or without skim milk (0.1%).

Evaluation of compost extracts on detached bean leaves and young bean plants

The filtrates of the extracts of each compost were initially tested on detached bean leaves. Drops (50 µl) of a spore suspension of *B. cinerea* containing 10^5 spores/ml of compost extract were placed on young detached bean leaves. Inoculated leaves were placed in Petri dishes on moistened blotting paper and incubated at 20°C for three days. Further tests were conducted on young bean plants at the two leaf stage. Ten punctures were made in each leaf with a hot needle and the compost extracts were applied by a hand sprayer. As soon as plants dried they were inoculated with a spore suspension of *B. cinerea* containing 10^6 spores/ml and incubated in a moist chamber for three days.

The number of bacterial cfus/ml was always much higher in the extracts obtained from peptone + dextrose amended composts than the other treatments.

Using detached bean leaves, all composts tested, either amended or not, were highly effective and protected from *Botrytis* almost completely. In contrast, compost extracts applied to wounded young bean plants were effective only when composts were amended before fermentation with dextrose + peptone.

Greenhouse experiments

So far three greenhouse experiments have been conducted. In the first and second experiments, extracts from all the composts were evaluated. In the third experiment, extracts obtained from the fermentation of sheep manure compost, with or without skim milk, integrated with iprodione was tested (Table 1).

The data obtained from the first and the second experiments showed that compost extracts were effective both against grey mould and ghost spot infection of tomato. Those of the third experiment (Table 1) indicated that the number of infected sites of the plots treated with sheep compost extracts, either integrated with iprodione or not, was significantly less than in the control plots. Also the infected sites of the extract + iprodione treated plots were less than in the iprodione alone or extract alone treated plots.

Bacterial microflora of the phylloplane in the extract treated plots was much higher than in the control. Finally, data obtained from the second experiment indicated that, except for straw manure compost, *Botrytis* was less frequently isolated from flowers in the compost treated plots than from the flowers of the control plots.

Table 1. Effect of sheep compost extract integrated with iprodione on tomato grey mould and microflora of leaf surface and flowers

Treatments	Infected sites/plot	Bacteria (cfu/cm^2 leaf surface (x10^7))	*Botrytis* on flowers	*Cladosporium* on flowers
			(Colonies/plate)	
Compost extract	19.0 *b**	12.0 *c*	2.0 *a*	22.0 *ab*
Compost + 2% milk extract	17.0 *b*	30.0 *a*	0.5 *a*	20.0 *ab*
Compost extract + iprodione	11.5 *c*	26.0 *b*	3.0 *a*	20.0 *ab*
Compost + 2% milk extract + iprodione at 5 week intervals	11.0 *c*	24.0 *b*	1.0 *a*	25.0 *a*
Iprodione at 5 week intervals	18.0 *b*	1.5 *d*	2.0 *a*	18.0 *b*
Water only control	22.5 *a*	1.1 *d*	4.0 *a*	18.0 *b*

* Different letters indicate statistically significant differences at the 5% level

Factors involved in the effectiveness of compost extracts against B. cinerea

Extracts treated in several ways were tested against *B. cinerea* on detached bean leaves (Table 2). The removal of the solid material by filtration through a single or a double layer of cheese cloth or by centrifugation did not reduce the effectiveness of the compost extracts. However, when micro-organisms in the extracts were removed by filtration or destroyed by steaming, their effectiveness was dramatically reduced (Table 2).

Table 2. Protection (%) of detached bean leaves against *B. cinerea* with compost extracts subjected to various treatments

Treatments	Filtration		Low-speed centrifugation	Sterilisation	
	Single	Double		Steam	Filter
Cattle compost	100 *a**	100 *a*	100 *a*	8 *a*	0
Sheep compost	100 *a*	100 *a*	100 *a*	2 *a*	0
Chicken compost	99 *a*	100 *a*	96 *a*	0 *a*	0
Seaweed + chicken compost	100 *a*	100 *a*	100 *a*	10 *a*	0

* Different letters indicate statistically significant differences at the 5% level

Biocontrol by fungal antagonists

Fungal antagonists used in our studies were mostly obtained from the phylloplane of greenhouse crops. Several isolates of *Cladosporium* spp. and *Penicillium* spp., which are the major species of resident mycoflora of greenhouse grown plants around the year, were studied. In addition an isolate of *Trichoderma* provided by Dr Gullino (University of Torino) and an isolate of *Acremonium alternatum* were included in the study. All isolates were initially screened in the laboratory on detached bean leaves. Those most effective were further tested on young bean plants by the methods used for compost extracts. Finally, the most promising isolates were evaluated in experiments carried out using greenhouse grown tomatoes.

So far 115 isolates of *Cladosporium* spp. and 15 isolates of *Penicillium* spp. have been screened (Table 3). In the greenhouse, several of the antagonists were tested either alone or integrated with chemicals. In one of them Trichodex, a formulation of *Trichoderma harzianum* produced by Maktheshim-Agan company (Israel) was included. Table 4 shows that, despite their effectiveness in the laboratory, *Penicillium* sp. and *A. alternatum* were not effective against *B. cinerea* at spore concentrations of 5 x 10^6/ml. Only Trichodex reduced infection by about 50%. Table 5 shows that all isolates of *Cladosporium* were very effective and those of *Penicillium* moderately effective.

In addition, the influence of temperature and the time of application of three antagonists on PDA, cucumber slices and young bean plants on their effectiveness against *B. cinerea* was studied (Malathrakis & Kritsotaki, 1992). These data indicate that the earlier the application of the antagonists and the higher the incubation temperature, the stronger was the inhibition of *B. cinerea.*

Table 3. Effectiveness of several bacterial strains, isolated from composts and bacterial and *Cladosporium* strains, isolated from phylloplane, against *B. cinerea* on detached bean leaves

% protection	Bacteria		*Cladosporium*	*Penicillium*
	Composts	Leaf surface	Leaf surface	
0-50	29	27	57	6
50-70	3	5	13	2
70-90	10	22	32	4
90-100	15	51	13	3
Total	57	105	115	15

Table 4. Effectiveness of antagonists integrated with iprodione against grey mould

	Treatments	% infected fruits	Infected sites/plot
A. alternatum	1 x 10^6	8.0 *a**	44 *a*
	5 x 10^6	5.2 *ab*	32 *bc*
	5 x 10^6 + iprodione 0.05%	1.6 *c*	11 *d*
Penicillium	1 x 10^6	5.9 *ab*	35 *abc*
	5 x 10^6	5.9 *ab*	33 *bc*
	5 x 10^6 + iprodione 0.05%	1.6 *c*	11 *d*
Trichodex	0.2%	3.1 *bc*	27 *c*
	0.2 % + iprodione 0.05%	1.7 *c*	11 *d*
Iprodione	0.05%	1.0 *c*	6 *d*
Control		6.2 *a*	41 *ab*

* Different letters indicate statistically significant differences at the 5% level

Table 5. Effectiveness of spore suspension (1 x 10^6 spores/ml) of several strains of *Cladosporium* and *Penicillium* against tomato grey mould in the greenhouse

Treatments	Infected sites/plot			
	29 Dec	2nd record	3rd record	4th record
Cladosporium 115	4.7 *a**	12.0 *b*	26.0 *cd*	14.3 *d*
Cladosporium 124	5.2 *a*	7.2 *b*	16.8 *d*	15.0 *d*
Cladosporium 132	6.0 *a*	5.7 *b*	18.0 *d*	17.0 *cd*
Penicillium 21	5.8 *a*	12.2 *b*	32.8 *bc*	31.7 *bc*
Penicillium 32	5.8 *a*	15.8 *b*	39.5 *b*	35.7 *b*
Water only control	4.0 *a*	24.0 *a*	54.3 *a*	64.3 *a*

* Different letters indicate statistically significant differences at the 5% level

Biocontrol by bacterial antagonists

Bacterial antagonists were obtained either from the phylloplane of greenhouse plants by washing leaves or flowers and plating washings on Petri dishes, or from compost extracts by plating dilutions on Petri dishes. A large number of isolates were obtained and 162 of them tested on detached bean leaves, by the method used for compost extracts, at the concentration of 10^8 cfu/ml. Several of them were highly effective.

Discussion

The data obtained during this six year study indicated that compost extracts alone applied at 7 day intervals reduced grey mould and ghost spot of tomato up to 60%. Similar effectiveness was obtained by McQuilken *et al.* (1993) against grey mould of lettuce. Moreover, compost extracts alternated with iprodione at 4 week intervals protected tomato plants better than either of them alone. Skim milk considerably increased bacterial numbers in the extracts but then the extracts were toxic to tomato plants. Other additives might be more suitable. Protection obtained by compost extracts was due directly or indirectly to the microbial population developed during compost fermentation. A high bacterial population is required to obtain adequate control of grey mould. In conclusion, our experiments provide convincing evidence that compost extracts were moderately but consistently effective against grey mould in greenhouses.

Among the fungal and bacterial antagonists tested there were isolates which were highly effective in the laboratory and some fungal antagonists moderately effective in the greenhouse as well. Trichodex, which is already used in some countries, is also moderately effective.

As with most biocontrol agents, none of those that we tested was effective enough to be accepted by the growers as a regular control means. However, our data indicate that compost extracts can be used to reduce the rate of the iprodione applied in the greenhouse against grey mould. Similar data has been obtained by Elad *et al.* (1992) for Trichodex.

Acknowledgements

Part of this work has been funded by the E.U. through CAMAR No. 8001-CT90-0019 project. We are grateful to Dr B. Seddon, Aberdeen University, Department of Agriculture, Scotland, for helpful comments on the manuscript.

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ROLE OF BIOLOGICAL CONTROL IN THE CYCLE OF *SCLEROTIUM CEPIVORUM* INOCULUM

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Abstract

Reductions in the size of sclerotial populations in soil form the basis for the control of *Allium* white rot (*Sclerotium cepivorum* Berk.) irrespective of whether the methods used to achieve this are chemical or biological. Targeting biological control measures at the renewal phase of inoculum, that is at sclerotia produced on the base of the infected plant, would have the advantages of maximising contact between biological control preparations and sclerotia, and of making use of sub-lethal soil temperatures to weaken sclerotia and increase microbial invasion. Biological control of inoculum renewal, in combination with crop management techniques which maximise host-induced sclerotial germination, and hence deplete primary inoculum, has promise for the long-term control of white rot.

Introduction

Allium white rot (*S. cepivorum* Berk.) is the most important soil-borne disease affecting green and dry bulb onions, garlic and leeks in temperate areas of the world (Entwistle, 1990). Primary inoculum is in the form of sclerotia which are produced on the host stem base and become incorporated into soil at harvest. Sclerotia germinate in response to host root exudates (Coley-Smith, 1960); the emerged hyphae infect roots and spread to neighbouring healthy plants. The fungus has no known sexual stage.

Control measures are based on the eradication of sclerotia from soil prior to planting, or on the protection of the plant with fungicides (Entwistle, 1990). Neither method is satisfactory in the UK due to such factors as enhanced degradation of active ingredient, lack of regulatory Approval of commercial products, excessive variability in the standard of control, or expense; commercial cultivars lack host resistance.

Reductions in the size of sclerotial populations in soil have the dual effect of decreasing white rot incidence and increasing the effectiveness of control measures, the latter presumably by reducing 'infection pressure' (Entwistle, 1986; Crowe *et al.*, 1980). This paper discusses the value of biological control to limit inoculum renewal and crop management techniques to maximise inoculum depletion.

Cyclic changes in inoculum

Sclerotial populations comprise a **basal population** present in soil at planting, a **new population** produced on the infected plant, and a **renewed basal population** resulting from the mixing of new and residual basal populations at harvest. Basal populations follow a

natural cycle of depletion by host-stimulated germination and renewal by the production of new sclerotia on infected plants (Figure 1). Studies by the author have shown sclerotial production to be up to 5,000 sclerotia per plant with germination about 80% within onion root systems and 50% between rows of onions.

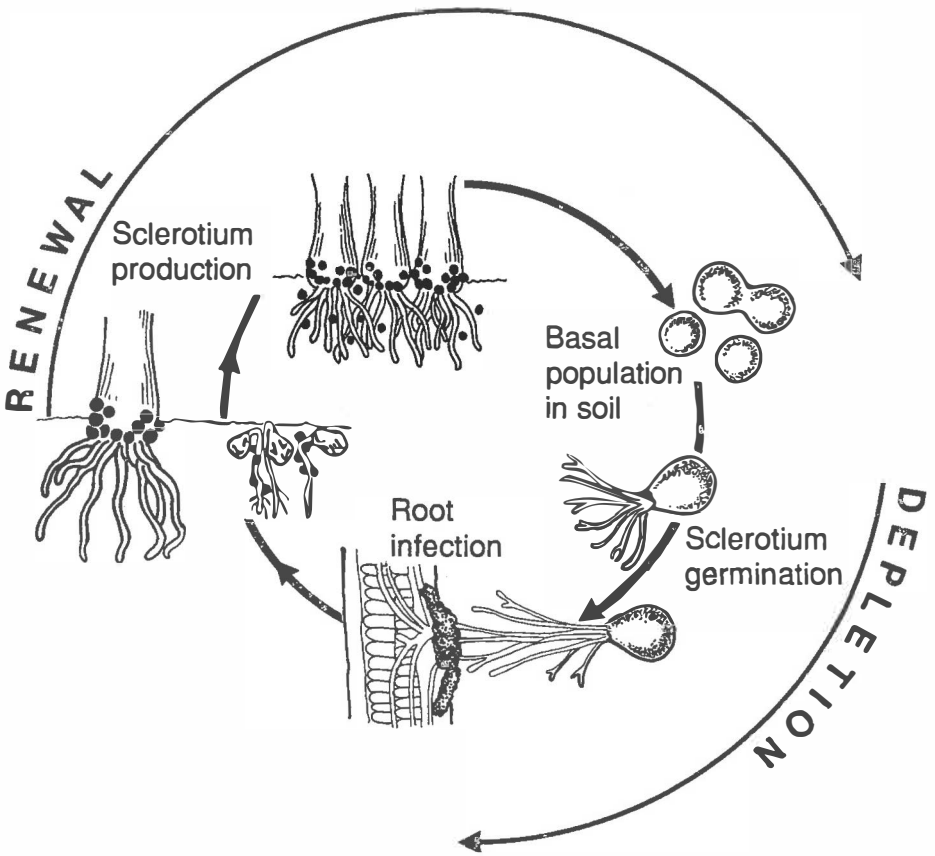

Fig. 1. *Sclerotium cepivorum* inoculum cycle

Opportunities for the biological control of white rot

The early promise of biological control to eradicate sclerotia in the basal population has not been sustained, possibly because of the practical difficulties of ensuring contact between biological control preparations and sclerotia. Similarly, a lack of success with seed treatments may be due to the requirement for prolonged protection which can be up to 9 months in overwintered onion crops (Entwistle, 1988).

As an alternative, biological control could be targeted at inoculum renewal. Two features of the pathogen provide opportunities for such an approach: (1) the accessibility of sclerotia to treatment as a consequence of their production on the stem base, and (2) the sub-lethal temperatures, commonly >40°C, which occur at the soil surface and which weaken sclerotia (Tables 1, 2) (Entwistle & Munasinghe, 1990). Weakening is evident as a decrease in the percentage viability of sclerotia, an increase in colonisation by microorganisms when tested on agar, a decrease in the percentage host-stimulated sclerotial germination and an increase in decay when tested in soil. There are opportunities to target sclerotia from initiation to maturity, and before or after exposure to sub-lethal temperatures. Treatment at the renewal stage of inoculum would also ensure that the microorganisms become incorporated into soil with the sclerotia and, hence, provide an opportunity for biological control to continue.

Table 1. Effects of temperature on *S. cepivorum* sclerotia

Temperature x duration (°C x days)	Sclerotia tested on agar		Sclerotia tested in soil	
	% viability	% colonisation by microorganisms	% germination	% decay
15 x 5	95	0	70-90	6
35 x 3	98	10	81	19
35 x 7	97	10	58	15
40 x 1	32	10	44	5
40 x 2	<1	50	15	34

Table 2. Temperatures at site of sclerotial production on onion base

July '94	Time (mins.) at different temperatures					Temperature (°C)	
	>25°C	30	35	40	45	max.	min.
12-13	660	300	60	0	0	37	17
16-17	630	480	300	90	0	40	12
19-20	650	540	400	290	90	47	14

Future research

The depletion of the basal sclerotial population by host-stimulated germination, and the renewal with sclerotia produced during infection are the two most important factors governing white rot control. Depletion of the basal population occurs naturally and there may be further scope by growing crops at planting densities and configurations which maximise occupation

of the soil by roots and, hence, maximise the proportion of the basal sclerotial population influenced by stimulatory root exudates. Ultimately, the success of biological control and crop management strategies for reducing sclerotial populations is likely to be governed by the ability to limit inoculum renewal.

Microorganisms, particularly *Trichoderma* spp., are often found on decaying, newly-produced sclerotia, suggesting that 'natural' biological control already operates. Such resident microorganisms should be compared with those from culture collections for their capacity to limit inoculum renewal, to survive in soil and to be selectively encouraged by appropriate crop management techniques. Comparisons should be at sub-lethal temperatures as observed in the field. It would also be prudent to investigate other methods of limiting inoculum renewal e.g. the physical removal of infected plant residues, or the treatment of disease patches by solarisation and fumigation (Entwistle, 1990).

Finally, the effects of reductions in sclerotial populations on white rot incidence should be investigated.

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CONTROL OF WHITE ROT OF GARLIC BY BIOLOGICAL AND PHYSICAL METHODS

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Abstract

White rot, induced by *Sclerotium cepivorum* Berk., is an important disease of Allium crops and severe attacks are frequent in garlic (*A. sativum* L.) fields in Spain. Soil solarization is one of the best approaches to control the disease in areas with suitable conditions. The application of antagonists such as *Trichoderma* spp. and *Gliocladium virens* to the soil offers other possibilities of non-chemical control. Four fungal isolates were selected by their antagonistic effects on *S. cepivorum in vitro*. Inoculum preparations were added to planting furrows in a plot artificially infested with *S. cepivorum*. Disease progress indicated a linear relationship between incidence of dead plants and time, with rate of disease progress significantly lower only for plots to which isolate JR2 of *T. harzianum* had been applied. These had an average final incidence of dead plants 13.6% in comparison to 33.4% for the untreated plots. Yields were increased by 33% because of treatment with *T. harzianum* JR2.

The application of *T. harzianum* JR2 to a naturally infested field was compared with soil solarization for 1 month and to the combination of both treatments (addition of antagonist in September) as well as the double application of *T. harzianum* in September and December. Soil solarization reduced pathogen inoculum to undetectable levels, resulting in very low rates of disease progress and yields ca. 150% higher than the controls. The application of *T. harzianum* to unsolarized plots brought about a reduction of disease progress. Corresponding garlic yields were intermediate between those for treatments involving soil solarization and that of untreated control.

Introduction

Each year garlic (*Allium sativum* L.) crops are grown over an average of 33,000 ha in Spain. White rot (WR), caused by *Sclerotium cepivorum* Berk., is the most important disease of *Allium* spp. which results in considerable yield losses, mainly in furrow-irrigated fields. The longevity of sclerotia of *S. cepivorum* in the soil (Coley-Smith, 1987) reduces the efficacy of short rotations with non-*Allium* crops as a control measure. Other possibilities of disease control include physical and chemical treatments to eradicate the pathogen in the soil (Coley-Smith, 1987; Entwistle, 1990). Soil solarization has proved to be fully satisfactory, bringing soil populations of the pathogen to negligible levels in areas with the appropriate weather conditions (Basallote-Ureba & Melero-Vara, 1993; Satour *et al.*, 1989) and provides a partial control in other areas (Porter & Merriman, 1985). Soil fumigants and germination stimulants give a good control over WR (Coley-Smith & Parfitt, 1986; Crowe *et al.*, 1990; Davies & Coley-Smith, 1990). The application of fungal and bacterial antagonists to the soil allows the possibility of disease control without the use of chemicals. Besides being environmentally

sound, it can be used once the crop is established. Among the reported microorganisms providing biocontrol of *S. cepivorum*, the most effective seem to be those that have an hyperparasitic mode of action such as *Trichoderma* spp. and *Gliocladium virens* (Abd-El-Moity & Shatla, 1981; De Oliveira *et al.*, 1984; Stewart & Kay, 1990). *Bacillus subtilis* was also considered an effective biocontrol agent (Utkhede & Rahe, 1983).

The objectives of this work were: 1) the selection of biocontrol agents effective against *S. cepivorum in vitro*; 2) assessment of the selected antagonists to control white rot of garlic under field conditions; 3) comparison of the control achieved by the antagonists with soil solarization.

Selection of antagonists *in vitro*

Dual culture tests on PDA (Royse & Ries, 1978; Jackson *et al.*, 1991) were carried out with one isolate of *S. cepivorum* and 22 fungal isolates (mainly from non-viable sclerotia of the pathogen) and six isolates of *B. subtilis*. Inhibition (%) of radial growth of *S. cepivorum* colonies was calculated for each isolate after 1-wk incubation. Ten days later, inhibition of the formation or maturation of sclerotia of the pathogen and occurrence of barrage zones in the dual cultures were recorded. Slide mounts with the pathogen and the potential antagonist were prepared to observe hyphal interactions that could indicate mycoparasitism of *S. cepivorum* (De Oliveira *et al.*, 1984).

Growth of *S. cepivorum* was only significantly inhibited when paired with either of four isolates of *T. harzianum*, one of *G. virens* and two unidentified fungal isolates. The highest inhibition occurred in the dual cultures with isolates JR2 and V1 of *T. harzianum*, both obtained from sclerotia from two contiguous fields in Vega de Granada, Spain. One isolate of *T. koningii* and one of *Dichotomophthora* sp. inhibited the formation and maturation of sclerotia, respectively. Hyphae of isolates JR2 and V1 of *T. harzianum*, *G. virens* and one of the two unidentified fungi coiled around the mycelium of *S. cepivorum*.

Biological control of white rot of garlic

A field experiment was conducted in an artificially infested field in Córdoba, Spain, aimed at comparing the effectiveness of applying four of the fungal isolates previously selected by tests *in vitro*. Inoculum of isolates JR2 and SR2 of *T. harzianum*, and those of *T. koningii* and *G. virens* was produced on a wheat bran: peat: water (5: 3: 2, w) substrate. It was incorporated into the planting furrows (11 g/m) immediately before garlic (ecotype "rojo de Cuenca") was sown in December 1992. Sequential observations on the incidence of plants killed by *S. cepivorum* were done from early March until harvest (June, 1993). Total yields were compared among the different treatments and effect on quality studied.

Disease incidence increased linearly with time (days) after planting in all the treatments. However, rates of disease progress were only 37 and 70% that of the untreated control in plots to which isolates JR2 of *T. harzianum* and *G. virens* had been applied, respectively. Consequently, yields were 34 and 15% higher than that of the control. The application of the two other fungal isolates (one of *T. koningii* and one of *T. harzianum*) did not show any beneficial effect in relation to disease control or garlic yields.

Comparison of soil solarization and addition of *T. harzianum* to the soil

Isolate JR2 of *T. harzianum*, was applied to a highly infested soil in Granada either twice (at preplanting in September and at planting in December, 1993), or once, at planting only. Inoculum production, rates and methods of application to the soil were similar to those in the previous experiment. These treatments were compared to soil solarization during July, 1993 and the integration of the latter with the application of the antagonist at planting. Disease progress was studied from mid March until harvest (June, 1994). Quantitative and qualitative yields were then compared among the different treatments.

Inoculum density of the pathogen in the arable layer of soil was nil in the plots immediately after solarization, and increased occasionally to extremely low levels during autumn. However, in the non-solarized plots, regardless of the treatment, inoculum density of the pathogen generally decreased during summer and increased again to levels similar or higher than those determined in early summer. Disease incidence progressed linearly with rates which were very low for treatments including soil solarization, intermediate for the treatments of antagonist into the soil and greatest for untreated control. Consequently, total yields were increased ca. 47% over the control in the treatments of application of *T. harzianum* and ca. 150% in the solarized plots. The greatest effect on bulb quality occurred in the treatment of soil solarization, with more than 80% of total weight in the two top quality categories, whereas in the untreated control only ca. 60% were in the top two quality categories. However, integration of soil solarization and addition of the antagonist did not result in any extra yield increase over the solarization treatment alone.

Discussion

The introduction of the antagonist did not seem to affect pathogen populations in the soil, at least in the short term. Application of antagonist into the infested soil at planting did reduce incidence of WR and increase yields, even though only small amounts of substrate in which *T. harzianum* was grown were added to the planting furrow. This is an affordable practice under the crop situation in Spain, which would be even more interesting if a long-term effect could be achieved. Unfortunately, this possibility seems unlikely since no reduction in the pathogen inoculum density was observed after the application of *T. harzianum* into the furrows.

The eradication of *S. cepivorum* inoculum brought about by soil solarization under our regular summer conditions (Basallote & Melero, 1993) has been confirmed with a shorter period of solarization. Therefore, this is an excellent method of controlling WR and increasing total yields and their quality. These effects could be only partially achieved by the introduction of an antagonist as effective as isolate JR2 of *T. harzianum* which is likely suited to our environmental conditions.

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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF A *FUSARIUM ACUMINATUM* SUBSP. *ACUMINATUM* STRAIN, A PARASITE OF *ASPERGILLUS* SCLEROTIA

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Abstract

Biochemical and molecular investigations were carried out in order to characterize *F. acuminatum* subsp. *acuminatum* strain ITEM 994 isolated from an *Aspergillus* sclerotium and potentially useful for biological control. *In vitro*, the strain inhibited mycelial growth of some phytopathogenic and toxigenic fungi at a distance, including an aflatoxin producing strain of *A. flavus*. When grown on maize for 4 weeks at 25°C in the dark, *F. acuminatum* ITEM 994 produced the antibiotic enniatin B (EB). In *in vitro* assays, EB inhibited the growth of *A. flavus* as well as of other plant pathogens. Isozyme analysis showed strain ITEM 994 to be identical to other isolates of *F. acuminatum* subsp. *acuminatum* with different origin, and they all differed from *F. acuminatum* subsp. *armeniacum*. On the basis of ecological behaviour, metabolite production and antagonistic activity, *F. acuminatum* subsp. *acuminatum* appears to be a good candidate as biological control agent.

Introduction

Fungi of the genus *Aspergillus* have a world-wide distribution. Several *Aspergillus* species are food contaminants causing decay of agricultural commodities and accumulation of mycotoxins in colonized tissues. Usually *Aspergillus* infections begin in the field, where sclerotia buried in the soil form a source of inoculum for several years.

Sclerotia of *A. flavus* var. *flavus* (Link) Fr. and *A. flavus* var. *parasiticus* (Speare) Kurtzman *et al.* buried in soil have been reported to be colonized by fungi of the genus *Fusarium* (Wicklow *et al.*, 1993). We examined some strains isolated from *A. flavus* sclerotia and identified them as *Fusarium acuminatum* Ell. & Ev. *sensu* Gordon. Recently this species was split by Burgess *et al.* (1993) into two subspecies, *F. acuminatum* subsp. *acuminatum* and *F. acuminatum* subsp. *armeniacum* on the basis of distinctive cultural and morphological characters. In addition, a study on toxigenic variability in *F. acuminatum* pointed out some significant differences in secondary metabolite production, indicating two distinct groups, producing trichothecenes and enniatin B (Fig. 1), respectively (Logrieco *et al.*, 1992). Trichothecenes are considered mycotoxins responsible of potential health hazards for humans and animals, whereas enniatin B is mainly regarded as an antibiotic.

Using biochemical and molecular procedures, this study aimed to characterize an *F. acuminatum* strain isolated from an *Aspergillus* sclerotium which is potentially useful for biological control of *Aspergilli*. In particular, we investigated the production of bioactive metabolites which were possibly involved in the antagonism exerted by this *F. acuminatum*

strain towards *A. flavus* and other agriculturally important fungi. In addition, isozyme analysis was performed in order to characterize the antagonistic strain in relation to other strains of *F. acuminatum*.

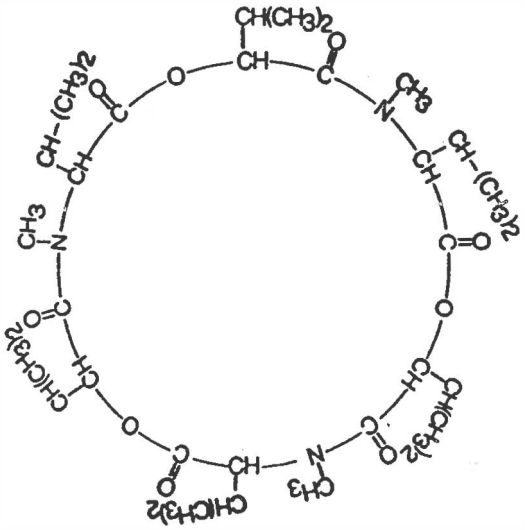

Figure 1. Structure of enniatin B

Materials and methods

Origin of F. acuminatum *ITEM 994*

The strain of *F. acuminatum* used in this study was originally isolated from an *Aspergillus* sclerotium from soil by Dr. D.T. Wicklow in U.S.A. The strain was isolated from a single germinated conidium, cryoconserved at -75°C and deposited in the culture collection of the Istituto tossine e micotossine da parassiti vegetali, under the accession number ITEM 994.

Antagonistic activity assay (dual plate)

F. acuminatum ITEM 994 was tested in a dual-plate assay against some phytopathogenic fungi, including an aflatoxigenic *A. flavus* strain (ITEM 9). The assay was carried out in Petri dishes (90 mm) containing 10 ml of PDA (Difco). The two fungi were inoculated simultaneously with 7 mm diameter discs of young cultures placed 5 cm apart. The percentage inhibition of growth (PI) was calculated as previously described (Whipps, 1987).

Production of bioactive metabolites

The toxigenic potential of the strain ITEM 994 was determined on autoclaved corn kernels maintained at 45% moisture. Cultures were incubated for 21 days at 25°C in the dark, then oven-dried at 50°C and finely ground. Extraction of active metabolites and analysis of

trichothecenes and enniatin B (EB) by high-performance-thin-layer-chromatography were performed as previously described (Logrieco *et al.*, 1992).

Antagonistic activity of EB

In order to evaluate the role of EB in the antagonistic activity of *F. acuminatum* ITEM 994, a methanolic solution of purified EB containing 5.0 µg of active compound per µl of methanol was assayed by paper disk assay. Ten µl of the EB solution were placed onto 6 mm disks (Difco, Detroit, USA) using a micropipette. After solvent evaporation, the disks were laid on the agar surface in Petri dishes (90 mm) containing 7 ml of PDA, and then the dishes were sprayed with a conidial suspension of the following test micro-organisms: *A. flavus*, *F. moniliforme*, *Geotrichum candidum* Link., or with a bacterial suspension of *Bacillus subtilis* (Gram+) or *Pseudomonas glycinae* (Gram-). The inhibition halo was measured after 24 h incubation. The experiments were replicated four times and the results were averaged.

Isozyme analysis

Lyophylized mycelium obtained from liquid cultures was ground in a mortar to a fine powder. Enzymes from 25 mg of mycelium were extracted in 0.3 ml Tris-HCl-PVP extraction buffer, pH 7.5 (Soltis *et al.*, 1983) and subjected to electrophoresis on 12.8% starch gel, according to the method described by Murphy *et al.*, (1990). Three different buffer systems were used: Tris-citric acid, pH 7.2, Tris-citric acid, pH 8.0 (Soltis *et al.*, 1983) and Tris-borate-EDTA, pH 8.6 (Goodwin *et al.*, 1993). After electrophoresis, gels were sliced and stained for enzyme activity following published procedures (Soltis *et al.*, 1983).

Results

In dual plate assays, *F. acuminatum* strain ITEM 994 inhibited the mycelial growth of *A. flavus* ITEM 9 and other fungal genera including *Alternaria*, *Fusarium* and *Penicillium*. The inhibition of *A. flavus* was visible after 4 days of incubation, at a distance of about 1 cm between the growth fronts of the colonies, and was maximum after 7 days (PI=35%). *F. acuminatum* ITEM 994 produced EB in amounts of 150 mg/kg when grown on autoclaved corn kernels. In contrast, no trichothecene toxins were detected in culture extracts of this strain. *F. acuminatum* ITEM 994 showed a relatively slow growth on PDA (6.2 cm of colony diameter after 7 days at 25°C), produced a reddish-brown colour in the medium and formed a dense greyish-pink floccose mycelium. The bioassays of the antibiotic activity against some test fungi and bacteria showed that all test organisms were inhibited by EB. *B. subtilis* (Gram+) proved to be the most sensitive bacteria (inhibition halo of 24.7 mm), whereas *P. glycinae* (Gram-) was about three times less sensitive (7.2 mm). Among the fungi tested, *A. flavus* appeared to be more inhibited by EB (7.5 mm) than *F. moniliforme* and *G. candidum* (6.2 and 6.3 mm, respectively). Isozyme phenotype (combination of electromorphs) of *F. acuminatum* ITEM 994 proved to be identical to *F. acuminatum* subsp. *acuminatum* ITEM 794 in all the eight enzyme systems used (Fig. 2). *F. acuminatum* subsp. *acuminatum* and *F. acuminatum* subsp. *armeniacum* could be distinguished on the basis of isozyme polymorphism of seven out eight enzymes (Fig. 2).

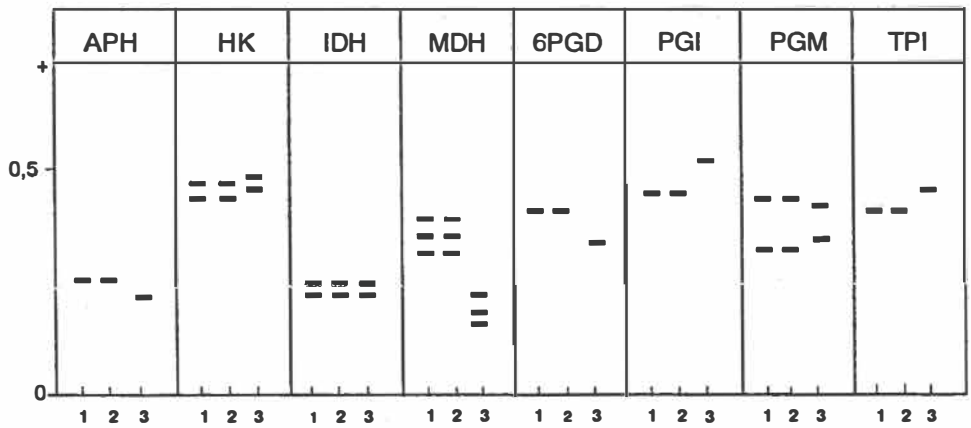

Figure 2. Isozyme phenotypes of *Fusarium acuminatum* ITEM 994 (1), *F. acuminatum* subsp. *acuminatum* ITEM 794 (2), and *F. acuminatum* subsp. *armeniacum* ITEM 796 (3) in eight enzyme systems: APH, acid phosphatase; HK, hexokinase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; TPI, triosephosphate isomerase.

Discussion

F. acuminatum strain ITEM 994, a mycoparasite of *Aspergillus* sclerotia, inhibited the mycelial growth of *A. flavus* and other phytopathogenic fungi in dual plate assays. The inhibition was visible before contact between the two fungi, indicating production and diffusion of active metabolites in the agar.

The cyclohexadepsipeptide antibiotic EB was identified in culture extracts of *F. acuminatum* ITEM 994. In *in vitro* assays, enniatin B inhibited the growth of a mycotoxigenic strain of *A. flavus* and other plant pathogens. *F. acuminatum* is also known to produce mycotoxins potentially hazardous for humans and animals, such as trichothecenes (Thrane, 1989). However, no trichothecene toxin was detected in culture extracts of *F. acuminatum* ITEM 994.

A recent study on cultural and toxigenic variability in *F. acuminatum*, showed that isolates of this species can be separated into two distinct toxigenic groups: EB producers, and trichothecene producers (Logrieco *et al.*, 1992). Isolates from soil, plant roots or rhizosphere were mostly EB producers. On the other hand, the trichothecene-producing isolates were mostly from the above-ground parts of plants. An investigation on antagonistic activity of several isolates belonging to these two groups showed that only the EB producing isolates were capable of inhibiting the growth of *A. flavus* and some other plant pathogens *in vitro*, and that antagonistic activity was mostly related to the amounts of antibiotic produced (data not shown).

In order to characterize the potential biocontrol strain better, especially in relation to the recent re-classification of *F. acuminatum* into the two subspecies *F. acuminatum* subsp.

acuminatum and *F. acuminatum* subsp. *armeniacum*, the isozyme phenotype of *F. acuminatum* ITEM 994 was compared with representative isolates of the two subspecies by starch gel electrophoresis. Isozyme analysis showed that the biocontrol strain and the isolate of *F. acuminatum* subsp. *acuminatum* were identical, and both differed from *F. acuminatum* subsp. *armeniacum* in electrophoretic phenotype of seven out of eight enzyme systems used in this study. These findings have been confirmed by isozyme analysis of 41 isolates of different origin belonging to both subspecies (Altomare *et al.*, unpublished).

Wicklow (1985) observed that the physiological attributes and secondary metabolites are ecologically relevant characters that define the fungal niche. From an ecological point of view, antibiotics produced by soil fungi could be regarded as chemical weapons providing competitive advantage. Although it is well established that antibiotic production in culture is not sufficient to attribute a role to them in the mechanism of biocontrol, it seems noteworthy that antagonism is a specific characteristic of the EB producing strains of *F. acuminatum* subsp. *acuminatum*. On the basis of ecological behaviour, metabolite production and antagonistic activity, *F. acuminatum* subsp. *acuminatum* appears to be a good candidate as biological control agent of sclerotia-forming *Aspergilli* and other soil-borne phytopathogenic fungi.

Acknowledgments

We thank Dr. D.T. Wicklow (National Center for Agricultural Utilization Research, Illinois, USA) for supplying the *F. acuminatum* strain used in this study.

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TALAROMYCES FLAVUS AS A POTENTIAL BIOCONTROL AGENT FOR CONTROLLING *VERTICILLIUM DAHLIAE* IN POTATOES

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Abstract

In two independent field experiments *Talaromyces flavus* reduced stem infection and stem colonization by *Verticillium dahliae* at low, but not at high densities of the pathogen in soil. Although, the population density of *T. flavus* in soil did not decrease the second year after application, the antagonist did not suppress stem infection or colonization by *V. dahliae* in the second year.

Introduction

V. dahliae is the primary pathogen involved in early dying (Verticillium wilt) of potato in the Netherlands (Bollen *et al.*, 1989). In relatively dry years, Haverkort *et al.* (1989) observed yield losses of 25%. The disease can be controlled by soil fumigation but, as expressed in the Multi Year Crop Protection Plan, the policy of the Dutch government is to reduce substantially the use of chemicals, especially of soil fumigants. Therefore, other control methods are required.

Talaromyces flavus is a potential antagonist for the biocontrol of a range of soil-borne plant pathogens, such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Verticillium dahliae* (Boosalis, 1956; Marois *et al.*, 1982; Davis *et al.*, 1986; Fravel *et al.*, 1986; Tjamos & Paplomatas, 1987; Madi *et al.*, 1991; McLaren *et al.*, 1994). The fungus is able to colonize the rhizosphere (Marois *et al.*, 1984) and to spread to new roots (Fahima & Henis, 1990). It multiplies more on roots infected with *V. dahliae* than on those without infection with this pathogen (Fahima & Henis, 1990). In contrast to most antagonists, *T. flavus* is resistant to dry conditions and, incorporated in a clay formulation, it has a long shelf life (Nagtzaam & Bollen, 1994). The aim of the present research was to evaluate the antagonist as a biocontrol agent against *V. dahliae* in field experiments in the Netherlands.

Experimental

The efficacy of *T. flavus* was studied in two field experiments. In Exp. 1a and 2, the soil was infested by applying a density of 50 microsclerotia g^{-1} and 10 or 30 microsclerotia of *V. dahliae* g^{-1}, respectively. In both experiments, the soil was amended with 43 kg ha^{-1} of a product prepared by grinding alginate-wheat bran granules containing ascospores of *T. flavus*. The granules were ground to improve distribution of the antagonist and increase the chance of contact between the antagonist and pathogen during the growing season (Nagtzaam & Van den Bosch, 1994). Besides the application of the antagonist to soil, seed

potatoes were treated with ascospores mixed in talc powder. The products contained a mixture of ascospores of three strains of *T. flavus*. In order to study the long-term effect of *T. flavus*, the plots of Exp. 1a were planted with potatoes in 1994, the second year after application, without reinoculation of pathogen or antagonist (Exp. 1b).

In all experiments, densities of *V. dahliae* and *T. flavus* in soil, and infection and colonization of stems by *V. dahliae* were assessed. Fresh and dry weight of tubers, leaves and stems were assessed by sub-sampling plots in Exp. 1a.

Results and Discussion

In the plots treated with *T. flavus*, the fungus was recovered from roots of potato plants and from soil samples (Table 1). In Exp. 1a and 2, it reduced stem colonization by *V. dahliae* in plots that had been infested with the pathogen at a low density (Table 2).

Table 1. Populations of *Talaromyces flavus* in soil and on potato roots in three field experiments

Treatment[a]	In soil (c.f.u. per g of soil)			On roots (c.f.u. per m root)	
	Exp. 1a 1993	Exp. 1b 1994	Exp. 2 1994	Exp.1a[c] 1993	Exp. 2[c] 1994
Co			2.3 *ab*		0 *a*
Cb	0 *a A*[b]	3 *a B*	7.7 *bc*	0.1 *a*	0 *a*
Tb	59.4 *c A*	66 *c A*	9.3 *c*	5.6 *b*	0.6 *a*
Vl			0.7 *a*		0 *a*
VlCb	7.8 *ab A*	4.7 *a A*	3.0 *abc*	0.1 *a*	0 *a*
VlTb	49.0 *c A*	51.7 *bc A*	7.7 *bc*	3.7 *b*	1.7 *a*

[a] Co = control; Cb = bran plus alginate and talc powder; Tb = *T. flavus*, bran plus alginate and talc powder; Vl = low level of *V. dahliae*; VlCb = low level of *V. dahliae*, bran plus alginate and talc powder; VlTb = low level of *V. dahliae*, *T. flavus*, bran plus alginate and talc powder.

[b] Different letters denote significant diffences within a column via Duncan's Multiple Range Test, P=0.05; capital letters are used to compare treatments at different times across rows

[c] 40 days after planting seed potatoes

Table 2. Effect of *Talaromyces flavus* on stem infection and colonization by *Verticillium dahliae* in three field experiments

Treatment[a]	Colonization (c.f.u. per 0.2 ml sap)			Infection (% infected stem segments)		
	Exp. 1a[d] 1993	Exp. 1b[d] 1994	Exp. 2[d] 1994	Exp.1a[c] 1993	Exp. 1b[d] 1994	Exp. 2[d] 1994
Co			0.3 *a*	19 *a*		10 *a*
Cb		1.3 *a*	0 *a*	19 *a*	45 *a*	8 *a*
Tb		6.8 *a*	0.3 *a*	12 *a*	51 *a*	27 *ab*
Vl			128.6 *d*			73 *d*
VlCb	110 *a*[b]	16.6 *a*	25.1 *ab*	66 *b*	36 *a*	68 *cd*
VlTb	58 *b*	15.6 *a*	24.5 *ab*	66 *b*	39 *a*	43 *bc*

[a] [b] See footnote, Table 1

[c] 83 days after planting seed potatoes

[d] 100-110 days after planting seed potatoes

In Exp. 1a, fresh weight of leaves was significantly higher ($P \leq 0.05$) in plots treated with *T. flavus* than in those without the antagonist. In Exp. 2 the reduction in stem infection and colonization was not followed by a decreased yield depression by *V. dahliae* (Table 3). Colonization of the roots by the pathogen was not affected (Table 4). Infection with *V. dahliae* was not suppressed in plots with a high pathogen density (data not shown). It is concluded that *T. flavus* has at least some potential to control *V. dahliae* in the field as the antagonist reduced stem infection and colonization in two independent experiments. However, this reduction was not consistently followed by decrease of yield depression caused by the pathogen. The effectivity of *T. flavus* seems to be dependent on pathogen density as biocontrol was obtained at low but never at high densities. The density at which control is feasible is likely to depend on the environmental conditions during the growing season and the quantity and type of antagonist inoculum applied.

In Exp. 2, but not in Exp. 1, stem colonization was at the same level in the bran plus alginate treatment without *T. flavus* (V1Cb) as in the treatment with the antagonist (V1Tb) (Table 2). The difference might be due to the method of application. In Exp 1., these additives and the inoculum of *V. dahliae* were applied separately, whereas in Exp. 2 they were added as a mixture. The mechanism(s) involved in suppression by bran plus alginate are not understood. It is hypothesized that unidentified antagonists are activated by wheat bran. An effect of bran is not exceptional. Ristaino *et al.* (1994) occasionally observed that a bran-only control formulation significantly reduced disease incidence. Another possibility is colonization by *T. flavus* in control bran-amended plots as *T. flavus* was detected at the same level as in the plots inoculated with *T. flavus* in Exp. 2 (Table 1).

Table 3. Effect of *Talaromyces flavus* on yield depression of potato caused by *Verticillium dahliae* in three field experiments

Treatment[a]	Yield tubers (tons per ha)		
	Exp. 1a 1993	Exp. 1b 1994	Exp. 2 1994
Co	36.8 *a*[b]		46.0 *bc*
Cb	33.5 *a*	34.1 *ab*	48.5 *c*
Tb	31.4 *a*	38.0 *c*	47.1 *c*
Vl			40.8 *ab*
VlCb	30.0 *a*	33.1 *a*	40.5 *ab*
VlTb	31.3 *a*	34.9 *ab*	37.9 *a*

[a] [b] See footnote, Table 1

Table 4. Populations in soil and on potato roots of the pathogen *Verticillium dahliae* in three field experiments

Treatment[a]	In soil (c.f.u. per g of soil)		On roots (c.f.u. per m root)		
	Exp. 1b 1994	Exp. 2 1994	Exp. 1a[c] 1993	Exp. 1b[c] 1994	Exp. 2[c] 1994
Co		1.0 *a*			0.3 *a*
Cb	1.5 *ab*[b]	1.6 *a*	0.9 *a*		0.4 *a*
Tb	0.8 *a*	1.3 *a*	0.2 *a*		1.1 *a*
Vl		10.8 *bc*			16.9 *bc*
VlCb	6.4 *c*	5.9 *b*	3.5 *b*	3.5 *a*	9.4 *abc*
VlTb	3.7 *abc*	10.3 *bc*	1.5 *ab*	1.7 *a*	12.6 *b*

[a] [b] [c] See footnote, Table 1

In spring 1994, density of *T. flavus* in the plots that were inoculated before planting in 1993 was the same as the density after the original inoculation (Table 1). Apparently, the conditions during the growing season and during winter did not reduce the antagonist population. However, *T. flavus* did not reduce stem infection or stem colonization in the second year after application (Table 2). At both a low and a high density of the pathogen, tuber yield was not increased in plots that were inoculated with *T. flavus*.

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AGENTS AND STRATEGIES FOR THE BIOLOGICAL CONTROL OF *VERTICILLIUM DAHLIAE*

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Abstract

Four possible approaches for the biological and integrated control of *Verticillium dahliae* are discussed. These involve the use of biological control agents such as *Talaromyces flavus* targeted at different stages of the life cycle of the pathogen as well as combining such treatments with sublethal soil fumigation or solarization.

Introduction

Microsclerotia of *Verticillium dahliae* are able to survive in soil for several years under adverse environmental conditions in the absence of host plants and create severe problems for the control of Verticillium wilt (Wilhelm, 1955). Among thousands of Verticillium-susceptible hosts, solanaceous crops are particularly vulnerable to this disease. The pathogen is mainly controlled through the use of resistant cultivars (when available), soil fumigation as well as soil solarization. Alternative control strategies involving the use of biocontrol agents are currently under study and these are discussed below.

Strategies for control of Verticillium wilt

Antagonists inhibiting formation of microsclerotia on diseased plant tissues after plant death

Verticillium wilt of cotton is a useful model system for this work as *Verticillium dahliae* microsclerotia are formed in parasitized plant tissues when the plant dies and later are released when the plant tissues disintegrate in the soil. Microsclerotia are formed in the vascular system and in the phloem in all annual and even perennial hosts. Among the antagonists, the soil-inhabiting fungus *Talaromyces flavus* has demonstrated potential for control of Verticillium wilt on various crops through a combination of mechanisms including antibiosis, parasitism and competition (Fahima *et al.*, 1992; Fravel, 1988, 1989, 1992).

Stems of *Verticillium*-infected cotton plants were sprayed with *T. flavus* (isolate Tfl-1 resistant to benomyl; Katan, 1985) ascospore suspension (10^4 ascospores/ml plus the surface adhesive agral) after the harvesting period and were incorporated into the soil. *T. flavus* failed to prevent formation of microsclerotia during tissue disintegration. However four months after spraying, the antagonist was present on the sprayed cotton stem surfaces at levels equal to those achieved after spraying, indicating good survival and potential for the biocontrol of those newly-formed microsclerotia.

Antagonists affecting the survival of microsclerotia in plant tissues during host disintegration in the soil

To assess viability of microsclerotia from *T. flavus*-treated and untreated control tissues, individual microsclerotia were removed from infected cotton tissues and transferred by a needle onto a semiselective agar medium based on soil extract. Microsclerotia from control unsprayed cotton stems grew normally on the medium. Initial trials did not clarify whether this particular antagonist was able to interfere significantly with the survival of *Verticillium dahliae* microsclerotia when it was sprayed on the host surface. However, there was some evidence of reduced germinability of microsclerotia from *T. flavus*-sprayed plants and a reduction in the vigour of such microsclerotia, expressed as restricted and delayed formation of new microsclerotia by those transferred to culture media. In some further experiments, recovery of the antagonist from microsclerotia was checked by plating individual microsclerotia on a *T. flavus* semi-selective medium with 50 mg/l benomyl. *T. flavus* colonies were only seen on 10-15% of microsclerotia. More research is needed to evaluate better the potential of this particular antagonist.

Antagonists preventing root infection by microsclerotia of the pathogen

Talaromyces flavus has shown potential for biocontrol of *Verticillium dahliae* by preventing root infection in several hosts (Marois *et al.*, 1982; 1984; Fravel 1989). Previous work in the USA indicated that *T. flavus* preferentially colonizes rhizospheres of eggplant, potato and cotton (Marois *et al.*, 1982, 1984; Fravel, 1989) while in Greece, rhizosphere colonization of globe artichokes, olive and tomato has been reported (Tjamos & Paplomatas, 1988; Tjamos & Skretis, 1990; Tjamos *et al.*, 1991). However, the precise site of establishment of *T. flavus* has not yet been identified. The data of this particular study indicate that *T. flavus* colonizes the elongation zone of root tips of solanaceous hosts (eggplant, potato and tomato) rather than the general rhizosphere or bulk soil. This is of particular importance as *V. dahliae* infects roots just behind the growing tip (Huisman & Gerik, 1989). Significantly, the zone of elongation behind the growing tip is associated with high levels of exudation of nutrients such as glucose. *T. flavus* produces the extracellular enzyme glucose oxidase which releases hydrogen peroxide in contact with glucose which is highly toxic to the microsclerotia of *V. dahliae* (Kim *et al.*, 1990; Fravel & Roberts, 1991). Consequently, the colonization of the zone of elongation by *T. flavus* is strategic for protection of the infection court from *V. dahliae*.

This part of our study was undertaken to determine both the spatial distribution and preferential establishment of *T. flavus* in the root zone, as well as the effect of the delivery system on the spatial distribution of the antagonist. Thus, ascospores of *T. flavus* were used as inoculum, either in an aqueous suspension containing 10^6 ascospores/ml for drenching the root system of the plants or in alginate prills (Fravel *et al.*, 1985). Tomato was initially selected as a model plant while eggplants and potatoes were included later. Pots (7.8 cm diameter) with one-week-old tomato or eggplants received 0.5 g of prill containing *T. flavus* or were drenched at 2, 3 and 4 wks after seeding with 10 ml of 10^6 ascospores/ml. For potatoes, either 0.5 g of prill containing ascospores of *T. flavus* was placed under each seedpiece before planting or the pots with potato seed pieces were drenched with 10 ml of an aqueous suspension of 10^6 ascospores/ml of *T. flavus*, at 3, 4 and 5 wks after planting. Fifteen plants from each treatment were destructively sampled at each sampling time. Prill

treatments were sampled at 4, 6 and 8 wks after planting and drenched plants were sampled at 6, 8 and 10 wks after planting.

To assess colonization of root tips by *T. flavus*, 4-6 roots were selected from each of the plants sampled and sixty root tips were removed from each plant. Tips were suspended in 3 ml of sterile distilled water in a test tube and the tubes shaken for 1 min. The suspension was then equally divided among each of three Petri dishes containing a medium semi-selective for *T. flavus* (TF medium). Colonization of roots tips by *T. flavus* was recorded 7-10 days after plating. To assess colonization of roots by *T. flavus*, 4-6 roots were selected (approximately 30 cm of total length of roots). Root tips were removed and roots were cut into 0.4-0.6 cm long segments. The root segments were suspended in 3 ml of sterile distilled water and shaken and plated as described before. To assess populations of *T. flavus* in the rhizosphere soil, 1 g of soil surrounding the roots was collected and suspended in 9 ml SDW. The suspension was agitated for 1 min, diluted 1:10 in SDW and 1 ml plated onto each of three plates of TF medium. To assess presence of *T. flavus* in non-rhizosphere soil, soil from areas not containing roots was sampled, suspended and plated in the same manner.

Data indicated that the antagonist colonized the root system preferentially in comparison with rhizosphere or non-rhizosphere soil, as shown by the number of colonies originating from root tips or roots rather than the rhizosphere soil or non-rhizosphere soil (Table 1).

Table 1. Colonization of tomato roots and soil by *Talaromyces flavus*

	Colony forming units (cfu) of *Talaromyces flavus*/g soil or tissue following	
	Drenching application	Prill application
Root tips	1592	300
Roots	395	17
Rhizosphere soil	150	4
Non-rhizosphere soil	70	2

The distribution of colony forming units of *Talaromyces flavus*/g soil or tissue was similar among solanaceous hosts (potato, eggplant and tomato) (Table 2). Quantitative assessment of the populations originating from plated roots or root tips demonstrated preferential colonization of the roots for all three solanaceous hosts and for all sampling times, although the colonization became less pronounced at later sampling dates.

Percentage colonization of the plated roots and root tips was significantly greater in drenched plants reaching 40% for roots and 30% for root tips while with prill application the percentage colonization remained very low ranging between 6-7% of the plated roots and 2-4% in root tips. It should be stressed that populations of *T. flavus* recovered from root tips and expressed as colony forming units/g fresh weight were significantly greater than those recovered from the roots, rhizosphere and non-rhizosphere soil. Importantly, if colonization of root tips and roots were expressed on a surface area basis, then the difference would have been approximately 10-fold greater in favour of the root tips compared to the roots. These findings demonstrate the potential of *T. flavus* to become established on the root tips.

Table 2. Colonization of roots of solanaceous plants and soil by *Talaromyces flavus*

	Colony forming units (cfu) of *Talaromyces flavus*/g soil or tissue		
	Potato	Eggplant	Tomato
Root tips	1144	882	807
Roots	310	196	110
Rhizosphere soil	170	64	38
Non-rhizosphere soil	103	23	7

The experiment was repeated with eggplants by using *T. flavus*, strain Tfl-1, a benomyl-resistant biotype. Use of a benomyl-resistant biotype facilitated selective recovery of *T. flavus* on TF medium amended with 50 ppm (a.i.) benomyl. Plants were either drenched or prill were added to the soil, and after sampling, roots were thoroughly washed for 1 hour and plated. The benomyl resistant biotype *T. flavus* Tfl-1 and the wild-type colonized root tips and roots similarly, and were recovered from rhizosphere and non-rhizosphere soil at similar levels. Since vigorous washing of roots resulted in a significant decrease in colonized root tips, only a loose affinity of *T. flavus* to the root tips was indicated.

Current studies in Greece include isolation of endorhizosphere bacteria with possible antifungal activity against the pathogen. Several bacteria have been found that are able to grow inside the root tips of potato plants and some Gram positive isolates have been found that exhibit antagonistic activity against *Verticillium dahliae*.

Antagonists affecting the survival of microsclerotia weakened by sublethal solarization or sublethal fumigation

Microsclerotia of *Verticillium dahliae* were heated in water under one of three regimes to simulate soil solarization, and survival was compared with that of unheated controls (Katan & DeVay, 1991; Pullman *et al.*, 1981). Regime 1 consisted of 31°C for 10 h followed by 35°C for 14 h. Regime 2 consisted of 33°C for 10 h followed by 36°C for 14 h. Regime 3 consisted of 35°C for 10 h followed by 38°C for 14 h. Heating for 1-5 days with regime 1 did not affect the final number of microsclerotia which germinated but did affect the rate at which they germinated. Additional days of heating with regime 2 or 3 affected both the number of germinated microsclerotia and the rate at which they germinated (Freeman & Katan, 1988). Of the microsclerotia that germinated, colonies arising from heated microsclerotia grew slower, formed significantly fewer melanized microsclerotia, and melanization of these new microsclerotia was significantly slower than of those originating from the non-heated microsclerotia. When microsclerotia were heated as above, placed in nylon mesh envelopes and buried in raw soil with or without the biocontrol agent *T. flavus*, there was a synergistic interaction between the heating and the antagonist resulting in increased mortality of the microsclerotia. Sublethal heating and *T. flavus* can be combined to suppress Verticillium wilt of eggplant suggesting future opportunities for integrating either solarization or sublethal fumigation with thermotolerant antagonists.

Conclusions

Our studies concerning the inhibition of the formation of microsclerotia of *V. dahliae* and the effect of antagonists on the vitality of existing microsclerotia could result in a future mode of biological control of this pathogen. This work also provides evidence that *T. flavus* preferentially colonizes the root tips and the roots of various solanaceous hosts. The observation that microsclerotia could be weakened with sublethal heating combined with *T. flavus* suggests that combining either solarization or sublethal fumigation with the application of thermotolerant antagonists could be used to suppress Verticillium wilt in the field. These findings may also trigger relevant screens to evaluate other potential biocontrol agents with ability to establish on the root tips, the actual site of invasion by *V. dahliae*.

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OVERVIEWS

BIOLOGICAL CONTROL OF *BOTRYTIS* SPP.: OVERVIEW AND FUTURE APPROACHES

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Abstract

Key features associated with the successful biological control of *Botrytis* spp. on a range of crop plants are discussed. Areas identified for future study include genetic manipulation of antagonists, understanding modes of action, and improving formulations and survival of antagonists in the phylloplane.

Introduction

Among foliar pathogens, *Botrytis* spp. represent one of the most widely investigated in the field of biological control. This is probably due to the significant losses caused by grey mould, the frequency of Botrytis blights on many economically important crops and to the difficulties encountered in their chemical control (Gullino, 1992a).

The efficacy of *Trichoderma viride* used as a biocontrol agent against *Botrytis cinerea* was reported a long time ago (Wood, 1951) and since then, several attempts have been made to develop effective and reliable biocontrol agents against *B. cinerea* as well as against other species of *Botrytis*. However, these efforts have resulted in only one compound, Trichodex, based on *Trichoderma harzianum* strain T 39, being registered for use in some countries (Elad *et al.*, 1994).

Different strategies for biological control of *Botrytis* spp. have been exploited and these are related to the three major stages in the life cycle: infection, sporulation and survival. Biocontrol agents active against each of the stages in the life cycle have been identified and they have exhibited the following mechanisms of action:

- competition for nutrients;
- suppression of sporulation;
- mycoparasitism;
- induced resistance;
- production of antibiotics;
- production of enzymes

Mechanisms exploited in biocontrol

Competition for nutrients

Necrotrophic pathogens, such as species of *Botrytis*, generally use exogenous nutrients in order to germinate and grow on the plant surface before penetration. In this environment, they often have to compete with the phylloplane microflora for limited nutrient sources (Elad, 1993).

Successful control of *B. cinerea* on grapevine (Dubos *et al.*, 1978) and on other crops such as cucumber, tomato, strawberry (Elad & Zimand, 1992) through the use of *Trichoderma* spp. has been, at least partially, explained by the capability of such an antagonist to compete with the pathogen for its nutritive base. Also in the case of antagonistic yeasts and bacteria used against Botrytis rot of fruit, competition for nutrients may occur: *Cryptococcus laurentii* competes for carbon and nitrogen sources with the pathogen (Roberts, 1990).

Suppression of sporulation

Antagonists can be applied in order to suppress sporulation of *Botrytis* spp. on necrotic leaf tissue. This approach implies interactions in dead plant tissue (Fokkema, 1993). In this way, initial or subsequent inoculum can be suppressed, thus influencing the progression of epidemics. This strategy has been successfully attempted for the control of *Botrytis* spp. on onion and strawberry (Sutton & Peng, 1993; Köhl *et al.*, 1995). *Gliocladium roseum* applied to green and overwintered strawberry leaves previously inoculated with *B. cinerea*, suppressed sporulation of the pathogen after artificial killing of the leaf tissue (Sutton & Peng, 1993). Suppression of sporulation on dead plant tissue has the important feature of being based on a long interaction time between pathogen and antagonist (Fokkema, 1993).

Mycoparasitism

Although not very common, this mechanism of biocontrol was suggested, in some cases, as a possible explanation of the interaction between *Trichoderma* and *B. cinerea* on grape (Dubos, 1992).

Induced resistance

Spraying watery fermentation extracts of well composted organic materials provided control of *B. cinerea* on strawberry and beans (Tränkner, 1992; Weltzien, 1992) and tomato (Elad & Shtienberg, 1994). The mechanisms suggested are induced resistance and direct inhibition of the pathogen (Weltzien, 1992).

Production of antibiotics and inhibitory compounds

In some instances, control of *B. cinerea* by antagonistic microorganisms has been explained through the production of antibiotic compounds. A strain of *Bacillus brevis* was shown to be active against grey mould of chinese cabbage, due to the production of an antibiotic (Edwards & Seddon, 1992). Pyrrolnitrin, produced by *Pseudomonas cepacia*, is responsible for biocontrol of *B. cinerea* on apple, pear (Janisiewicz *et al.*, 1991) and rape seedlings. Phenylpyrroles have been recently developed as fungicides (Nyfeler & Ackermann, 1992).

However, antibiotic production is generally not considered as a positive feature, when considering treatments of edible plant parts, due to the possible presence of undesirable residues. Inhibitory compounds may also accumulate in the water extracts of compost, being at least partially responsible for their activity.

Enzyme production

In the case of *Pichia guilliermondii*, active against Botrytis postharvest fruit rot, a high level of β1,3-glucanase activity was detected when cultured on various carbon sources or on cell walls of several fungal pathogens (Wisniewski *et al.*, 1991). In the case of the combination *P. guilliermondii* - *Botrytis cinerea*, firm attachment of the yeast cells along with the production of hydrolases is considered responsible for the observed degradation of the fungal cell wall following interaction with the antagonist (Droby & Chalutz, 1994).

Achievements

Some selected biocontrol agents have been widely used in different situations, in the phylloplane or during storage of fruit, vegetables and flowers.

In the first case, application of *Trichoderma* spp. in the phylloplane led to a satisfactory control of grey mould on grape, tomato, cucumber, strawberry and apple (Tronsmo & Dennis, 1977; Tronsmo & Ystaas, 1980; Dubos, 1992; Elad & Zimand, 1992; Gullino, 1992b). However, in some cases, poor survival in the phylloplane resulted in unsatisfactory control (Elad, 1990). The use of biocontrol agents fits well in an integrated disease management approach: the alternation of chemicals and *Trichoderma* spp. resulted in very positive results on several crops.

In the case of postharvest applications, bacteria and yeast isolates controlled Botrytis rot on apple, pear and kiwi (Janisiewicz & Roitman, 1988; Roberts, 1990; Gullino *et al.*, 1991; Testoni *et al.*, 1993). *T. harzianum* was effective in reducing Botrytis rot on carrots during storage (Tronsmo, 1989).

Post-harvest use of biocontrol agents looks particularly promising on fruits, where the need to reduce the use of chemicals is strongest (Droby & Chalutz, 1994).

Flowers can be treated after harvest with biocontrol agents, in order to reduce damage caused by *B. cinerea* during storage and transit: several bacteria and yeast isolates, as well as *Trichoderma* proved effective and promising (Hammer & Marois, 1989; Elad *et al.*, 1993; Pasini *et al.*, 1995).

Future directions

In order to improve the efficacy of the known biocontrol agents against *Botrytis* spp. and to increase the number of available ones, there is a need for:

- better survival of biocontrol agents in the phylloplane;
- improvement of the biocontrol capabilities, possibly through use of genetic manipulation;
- better understanding of their mechanisms of action;
- more research in the field of formulation.

Acknowledgements

Research supported by a grant from Ministero delle risorse agricole e forestali (Progetto Finalizzato Lotta biologica e integrata. Sottoprogetto: Viticoltura).

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BIOLOGICAL CONTROL OF BLACK SCURF IN POTATO USING THE MYCOPARASITIC FUNGUS *VERTICILLIUM BIGUTTATUM*

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Abstract

The soil-borne pathogenic fungus *Rhizoctonia solani* AG-3 is the causal agent of the black scurf symptom (sclerotia of *R. solani*) on progeny tubers in Dutch seed potato growing. Agrochemicals, like herbicides and fungicides are currently employed to meet the standards of certified seed with respect to virus infection and black scurf, respectively. In an attempt to reduce agrochemical input in Dutch agriculture, Green-Crop-Harvesting (GCH) has recently been adopted as a mechanical alternative for the conventional, herbicide-based, haulm destruction method. GCH adequately prevents virus-transmission to the progeny tubers and allows antagonists to intervene with black scurf formation on progeny tubers.

From earlier work on seed tuber inoculation, the mycoparasitic fungus *Verticillium biguttatum* was known to potentially reduce disease symptoms by *R. solani* (Jager *et al.*, 1991). However, low soil temperatures at planting time may hamper the reliability of seed tuber treatments as a strategy to control black scurf. Application of *V. biguttatum* in GCH has shown to be a promising strategy as the fungal inoculum can be applied site-directed (on progeny tubers and below-ground plant parts) at sufficient spore-concentrations under conducive temperature and moisture conditions for efficient mycoparasitism. In addition, *V. biguttatum* has been shown to be compatible with a number of microbial antagonists against other pathogens, e.g. *Coniothyrium minitans, Trichoderma* spp. and antagonistic *Pseudomonas* spp., which opens a promising outlook for broad disease control in commercial crop rotations.

Recent *in vitro* studies on nutrition requirements and microscopic interaction with its host revealed that *V. biguttatum* behaved as a biotrophic mycoparasite. From germinating spores, it penetrates hyphae of *R. solani* and forms haustorium-like branches without killing the host cells. The haustoria support an external mycelial network of the mycoparasite. Later the mycoparasite sporulates, and the infected host cells die. On cellulosic substrata, *V. biguttatum* did not reduce the growth of *R. solani*, and often enhanced the rate of cellulose degradation. However, *V. biguttatum* drastically reduced the production of sclerotia by *R. solani*, often completely suppressing sclerotium formation when the mycoparasite infected only a localized region of the host colony. This distance effect is ascribed to the creation of a nutrient sink by the parasite, consistent with biotrophy. Suppression of sclerotium production may explain the success of application of *V. biguttatum* in biocontrol of black scurf on progeny tubers in GCH.

We will report on our data concerning biological control by *V. biguttatum* in the field and the supposed mechanism underlying black scurf control.

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RECENT ADVANCES AND FUTURE APPROACHES FOR THE BIOCONTROL OF *SCLEROTINIA* SPP.

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Abstract

In the last ten years, several approaches for biological control of *Sclerotinia sclerotiorum* have shown promise. Cultural control through use of soil solarization has proven successful in regions with high insolation but most effort has been focused on the use of sclerotial mycoparasites such as *Coniothyrium minitans*, *Trichoderma* spp. and *Sporidesmium sclerotivorum*. Advances have been made in production of inoculum of these mycoparasites but further work is still required to improve liquid fermentation procedures. Novel screening procedures for antagonists of *S. sclerotiorum* have also been developed with indications that soils suppressive to *Sclerotinia* may be valuable sources of these antagonists. Application of combinations of antagonists selected for use in soil, on crop debris, on seeds and on foliage could well prove fruitful in the future, particularly if integrated with reduced applications of fungicide. Most recently, genetic modification of plants for resistance to *S. sclerotiorum* has been achieved and this may prove a significant advance for control of this pathogen.

Introduction

Despite considerable research effort, *Sclerotinia* spp. remain some of the most intractable disease problems. These pathogens infect more than 350 species of plants worldwide and regularly cause major crop losses. No resistant cultivars of commercially grown, susceptible crops are yet available. Current cultural and physical control methods such as crop rotation are generally inadequate although soil solarization has provided successful control of *S. sclerotiorum* in areas with high insolation (Phillips, 1990) and use of ultraviolet-absorbing vinyl film has controlled *S. sclerotiorum* in greenhouses by preventing apothecial formation (Honda & Yunoki, 1977). Fungicide treatments may help control ascospore infections and prevent seed-borne carryover to some extent, but soil applied drenches are rarely efficient or cost-effective enough to control infections arising directly from sclerotia in the soil. Nevertheless recently, soil enrichment with S-H mixture (a formulated product for control of soil-borne plant pathogens) and calcium cyanamide effectively controlled *S. sclerotiorum* (Huang & Sun, 1991) indicating scope for more work in this area. Increasing costs of steaming, and environmental problems with the use of methyl bromide and partial soil sterilants, further emphasise the need for alternative biological control treatments for *Sclerotinia* disease. The aim of this brief overview is to outline areas of research where advances have been made recently and indicate possible future directions that could be taken.

Selection and screening of antagonists

The whole area of selection and screening of antagonists has now moved away from unrealistic studies involving *in vitro* tests performed on agar. Now, to optimise the chances of establishment, selection has focused on isolating antagonists from the eventual environment of use. For example, bacteria for subsequent application on sunflower seeds were isolated from rhizosphere soil (McLoughlin *et al.,* 1992) and those for application on foliage were isolated from the phylloplane or petals of several plants (Mercier & Reeleder, 1987; Inglis & Boland, 1991; Yuen *et al.*, 1991; Huang *et al.*, 1993). Alternatively, to optimise the chances of obtaining active isolates, antagonists have been isolated from sclerotia, *Sclerotinia*-infected plant material and *Sclerotinia*-suppressive soils (Adams, 1987; Whipps & Budge, 1990; Sandys-Winsch *et al.*, 1994). *Sclerotinia* suppressive soils have already yielded several new antagonists (Adams & Ayers, 1981; Huang & Kozub, 1991; Whipps *et al.*, 1993).

Similarly, screening now frequently involves the use of tests designed to mimic the part of the life cycle of the pathogen which has been targeted for control. For instance, seedling tests are used to screen antagonists designed to protect roots from mycelial infections (McLoughlin *et al.*, 1992; Sandys-Winsch *et al.*, 1994); petal, leaf or plant tissue based bioassays are used to assess antagonists to be applied to the phylloplane or crop debris (Mercier & Reeleder, 1987; Inglis & Boland, 1991; Gerlagh *et al.*, 1994); sclerotium based tests are also used to assess antagonists applied to soil (Whipps & Budge, 1990). Several antagonists selected and screened in this way have proven successful in controlling *S. sclerotiorum* in subsequent glasshouse or field trials, emphasising the value of these ecologically and environmentally based tests.

Screening for pesticide compatibility is also an area which is of considerable significance in terms of achieving commercial biological control of *Sclerotinia* spp. The ability to withstand exposure to fungicides used periodically would be a considerable advantage. Both the sclerotial mycoparasites *Coniothyrium minitans* and *Sporidesmium sclerotivorum*, which can provide reproducible control of *S. sclerotiorum*, have already been screened both *in vitro* and *in vivo* for such characteristics (Adams & Wong, 1991; Budge & Whipps, 1995).

Inoculum production and formulation of antagonists

Inoculum production of antagonists for use against *Sclerotinia* spp. is an area that has been largely neglected even for those antagonists used in large-scale glasshouse or field trials. Bacterial and fungal antagonists have been produced on agar or in shake flask cultures (Inglis & Boland, 1991; McLoughlin *et al.*, 1992; Huang *et al.*, 1993) and some fungal antagonists by solid-substrate fermentations on grain (Huang, 1980; Budge & Whipps, 1991); procedures for selecting optimal solid-substrates for production of *C. minitans* have recently been described (McQuilken & Whipps, 1995). However, methods for successful liquid fermentation of this fungus and *S. sclerotivorum* under commercially realistic conditions are still awaited. In contrast, both *Gliocladium virens* and *Trichoderma harzianum* which can act as mycoparasites of sclerotia of *S. sclerotiorum* have been produced in liquid culture and formulated as alginate pellets (Lumsden & Lewis, 1989; Knudsen *et al.*, 1991). The latter mycoparasite preparation was successfully used for the control of *S. sclerotiorum*. However, little other work on production and formulation of antagonists for control of *Sclerotinia* spp. has been carried out and this deserves further study.

Application of antagonists

The range of approaches taken for biological control of *Sclerotinia* spp. has increased recently, reflecting the growing awareness of the different parts of the life cycle of the pathogen that are open to attack. Initially, antagonists were applied to the soil in an attempt to destroy sclerotia before germination. Potentially, this should decrease both myceliogenic germination leading to infection of the roots or stem base, and apothecial germination, leading to ascospore release and infection of the aerial plant parts (Huang, 1980; Budge & Whipps, 1991). However, a range of other successful treatments has now been made. For instance, both bacterial and fungal antagonists have been applied on the phylloplane to occupy senescing tissues, particularly flowers. These are required as food bases by ascospores of *S. sclerotiorum* to allow infection to occur (Mercier & Reeleder, 1987; Yuen *et al.*, 1991; Inglis & Boland, 1991; Huang *et al.*, 1993). Further, spores of *C. minitans* have been applied in the field on the phylloplane of a range of crops exposed to *S. sclerotiorum* during the growing season (Gerlagh *et al.*, 1995). This has resulted in a decrease in *S. sclerotiorum* disease in subsequent years, reflecting the degradation of the sclerotia by *C. minitans* following incorporation of the plant debris into the soil. Similarly, application of spores of *C. minitans* to *S. sclerotiorum*-infected lettuce residues in the glasshouse also resulted in reduced *Sclerotinia* disease in subsequent crops (Whipps & Budge, unpublished).

It is also feasible to treat seeds or stored produce with antagonists to control *Sclerotinia*. For instance, bacterial cells applied to sunflower seeds resulted in increased seedling emergence when planted in soil containing sclerotia of *S. sclerotiorum* (McLoughlin *et al.*, 1992). In addition, the possibility of treating roots such as carrots or chicory to prevent damage by *S. sclerotiorum* during storage has been suggested (Gerlagh pers. comm.) and this topic deserves more study in the future.

Several additional points concerned with using antagonists are worthy of consideration. For example, in most commercial crop production systems, pesticides are widely used. Consequently, it would be useful for antagonists to be compatible with at least some of the more commonly used pesticides to allow integrated control to take place. This approach has been adopted with both *C. minitans* and *S. sclerotivorum* (Adams & Wong, 1991; Budge & Whipps, 1995). In addition, long term control following inundative applications could be desirable from a grower's point of view, and again, both *C. minitans* and *S. sclerotivorum* have exhibited this trait (Adams & Ayers, 1982; Budge & Whipps, 1991). The existence of soils suppressive to *S. sclerotiorum* which contain these antagonists further emphasises this point (Adams & Ayers, 1981; Huang & Kozub, 1991; Whipps *et al.*, 1993). Finally, the interactions between any applied antagonist, *Sclerotinia* spp. and the natural microbiota and microfauna deserve further study. For example, in Canada, larvae of the fungus gnat *Bradysia coprophila* consumed sclerotia of *S. sclerotiorum* and allowed further degradation by secondary mycoparasites such as *Trichoderma* spp., decreasing inoculum density of *S. sclerotiorum* in the soil (Anas & Reeleder, 1988). In the UK, sciarid larvae, collembolans and mites were demonstrated to transmit *C. minitans* from infected sclerotia of *S. sclerotiorum* to uninfected ones (Whipps & Budge, 1993; Williams & Whipps, 1995). The concept of combining soil microfauna and antagonists to improve biological control of *S. sclerotiorum* in the future is an exciting one.

Genetic modification

Genetic modification of antagonists used against other pathogens has already been achieved through protoplast fusion or transformation with heterologous genes such as chitinase (Harman *et al.*, 1989; Shapira *et al.*, 1989; Pe'er & Chet, 1990). It would seem to be just a matter of time before similar procedures are applied to antagonists active against *S. sclerotiorum*, although it remains to be seen whether these genetically modified antagonists will become commercially acceptable.

An alternative novel approach concerning the development of a debilitated pathogen has recently been reported (Boland, 1992). Hypovirulent isolates of *S. sclerotiorum* containing double-stranded RNA (dsRNA) were identified and this hypovirulence could be transmitted to virulent, recipient isolates by hyphal anastomosis and was associated with the transfer of dsRNA. The concept of utilizing transmissible hypovirulence for management of *S. sclerotiorum* is worthy of further study.

Another possibility is the genetic modification of susceptible plants. In initial work, Thompson *et al.* (1995) have produced transgenic oilseed rape plants containing an oxalate oxidase gene which are tolerant of exogenously supplied oxalic acid. In view of the primary role ascribed to oxalic acid in pathogenesis by *S. sclerotiorum*, this may well be the first step to the production of *S. sclerotiorum*-resistant plants. This area of work is bound to grow.

Induced resistance

Until recently, the possibility of induced resistance for *Sclerotinia* in plants following exposure of plant tissue to specific biological or physical (abiotic) elicitors had not been considered. However, Mercier *et al.* (1993) exposed carrot slices to UV-C (light of wavelength below 280 nm) and this caused the induction of 6-methoxymellein (a phytoalexin) and resulted in increased resistance of the tissue to *Botrytis cinerea* and *S. sclerotiorum*. This raises a whole new area of possible control of *S. sclerotiorum* in stored products and warrants further study.

Conclusions

Overall there have been many significant advances in the technologies associated with screening and application of antagonists, and several new avenues of research, particularly concerning genetic modification of various types, have been identified. It should only be a matter of time before one of these research areas is brought to fruition and a commercial product for the control of *Sclerotinia* spp. is on the market.

Acknowledgements

This work was funded by the Biotechnology and Biological Sciences Research Council and the Ministry of Agriculture, Fisheries and Food for England and Wales.

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