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**Study Group "Biological Control of Fungal and Bacterial Pathogens"**

**and**

EFPP

**The European Foundation for Plant Pathology**
**Working Group on Biological Control**

# NEW APPROACHES IN BIOLOGICAL CONTROL OF SOIL-BORNE DISEASES

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**D.F. Jensen, J. Hockenhull & N.J. Fokkema**

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# INTRODUCTION

This bulletin contains reviews and short research papers presented at the workshop "New Approaches in Biological Control of Soil-borne Diseases". This workshop, held at The Royal Veterinary and Agricultural University in Copenhagen, from June 30th - July 4th, 1991, was the first activity of the Working Group on Biological Control of the European Foundation for Plant Pathology (EFPP) and -also freshly started - the Study Group on Biological Control of Fungal and Bacterial Pathogens of the International Organization for Biological Control (IOBC/WPRS). The 4-day workshop with 68 contributions was attended by 78 scientists from 19 European countries, including several East-European countries, and two (former) soviet republics. One of the key speakers was from the USA.

The workshop was especially intended for young scientists and the programme was organised around seven review papers covering the use of non-pathogenic fusaria, pseudomonads, Trichoderma spp. and mycoparasites as antagonists in biological disease control, the improvement of biological control agents (BCA's) by genetic manipulation, improved delivery systems as well as research partnership between industry and state financed institutions.

The take-home lesson was that although great progress is being made at European universities and governmental institutions in discovering the possibilities of biological disease control, commercializing is still in its infancy. The main reason is that industry requires more information on BCA's regarding the possibilities for their mass production, formulation and delivery systems and consistent field performance than currently is offered by the non-profit institutes. Furthermore, consistency of field performance needs to be improved and the gap between basic science and its practical application will have to be narrowed especially if the declared policy of governments to reduce the use of, and the dependancy upon, chemical pesticides is to be fulfilled.

Thanks are due to the following organisations for supporting the workshop financially and thus helping to keep the cost of participation at a reasonable level:
- Danish Society of Plant Pathology
- European Foundation for Plant Pathology (EFPP)
- Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palaearctic Regional Section (IOBC/WPRS)
- Novo Nordisk A/S, Bagsvaerd, Denmark.

Concerning the future,the IOBC/WPRS Study Group and the EFPP Working Group are to continue their joint activities. A management committee consisting for the IOBC/WPRS of C. Alabouvette (France), Y. Elad (Israel) and N.J. Fokkema (Convenor IOBC/ WPRS, the Netherlands) and for the EFPP of G. Défago (Switzerland), J. Hockenhull (Denmark) and J. Whipps (UK) has been established.

This bulletin contains the camera-ready texts of the reviews and the research contributions presented at the workshop. Many of the research papers are preliminary and primarily aimed to disseminate progress in biological control research at a very

early stage. Editing was done very superficially to allow rapid publication. Therefore, minor faults were not corrected. We thank all authors for their contributions which are generally of a high standard.

The organization of the workshop and the editing of the proceedings would not have been possible without the assistance of Helge Green, Inge M.B. Knudsen, Danilo E. Paderes, Tina de Kleijn and Wilma Molhoek.

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# FUNGAL ANTAGONISTS

# METHODS TO BE USED TO STUDY THE COMPETITIVE INTERACTIONS BETWEEN PATHOGENIC AND NONPATHOGENIC *FUSARIUM* SPP IN THE RHIZOSPHERE AND AT THE ROOT SURFACE.

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## SUMMARY

Competition for nutrients in the soil and the rhizosphere, and competition for infection sites at the root surface are two mechanisms involved in biological control of fusarium wilts with nonpathogenic fusaria. To study these competitive interactions, it is necessary to develop accurate methods to either isolate or visualize the pathogenic and the nonpathogenic strains in soil, at the root surface or inside the root tissues.
Different staining methods with calcofluor or F.D.A. can be used to assess chlamydospore germination or growth kinetics of a given strain in raw soil or in disinfested soil. It is also possible to follow the population dynamics of a given population by isolation of a marked strain on selective media. Orange mutants and benomyl resistant strains have been used for this purpose. Another approach is based on the transformation of fusarium strains by the GUS gene system. This technique enables the visualization of hyphae at the root surface and the quantification of root colonization by the fungus. Immunoenzymatic techniques are also being developed to detect and quantify root colonization by pathogenic or nonpathogenic strains. However, to study competitive interactions between pathogenic and nonpathogenic strains, specific antisera are needed to differenciate the two types of strains. The advantages and limitations of these methods are discussed.

## INTRODUCTION

Studies of mechanisms of suppressiveness of fusarium wilts naturally occuring in suppressive soils showed the involvement of nonpathogenic fusaria (Louvet *et al.*, 1976). It was also demonstrated that the introduction of nonpathogenic *F. oxysporum* into conducive soils make them suppressive (Rouxel *et al.*, 1979). Therefore, many attempts have been made to control fusarium diseases of vegetables and flowers by application of nonpathogenic fusaria (Alabouvette, 1990). In this context, it is important to have a better understanding of the modes of action of the nonpathogenic fusaria. Three modes of action have been investigated (Mandeel and Baker, 1991) : 1) competition for nutrients in soil and rhizosphere, 2) competition for infection sites at the root surface, 3) induced resistance within the host. There are good arguments in favour of each of these modes of action but clear experimental evidence is missing. In fact, we face difficult methodological problems to assess the validity of these hypotheses. It is particularly difficult to study the competitive interactions between a pathogenic strain of *F. oxysporum* and the wild populations of *F. oxysporum* occuring in soil. In this specific case, the pathogen and the nonpathogens belong to the same species, and there is no simple technique enabling the two types of *F. oxysporum* to be distinguished. Therefore, we need to use specific markers or staining techniques to follow the behaviour of a given strain among other strains in raw soil or in model soil systems. Indeed to assess accurately the saprophytic competitive ability of a pathogen it is necessary to develop experimental procedures to enable the growth kinetics of the pathogen to be followed when confronted to a nonpathogen under well defined conditions ; the use of disinfested soil is often needed.

The aim of this paper is to review briefly the methods that have been used or are being developed to study the competitive interactions between pathogenic and nonpathogenic *F. oxysporum* in soil and at the root surface.

## 1 - STAINING METHODES

Several chemicals can be used to stain fungal propagules in soil or at the root surface. However, these staining techniques are not specific to *F. oxysporum* and their use is restricted to particular experimental conditions. In contrast, the immunoenzymatic methods should enable the specific visualization of a given species or even a given strain of fungus in soil. However, these methods have not been extensively used to study the competitive interactions between pathogenic and nonpathogenic *F. oxysporum*.

### 1.1 - CHEMICAL STAINING

Two types of chemicals are available : some stain the fungal wall, they are unable to distinguish between dead and alive propagules ; others become fluorescent after having been hydrolysed by the funGUS, they stain only the propagules that show metabolic activity.

#### 1.1.1 Use of calcofluor

Calcofluor white M2R New (American Cyanamid Co) is one of the most frequently used fluorescent brightners. It recognizes some components of the fungal cell wall and appears blue under U.V. light (Darken, 1962). It is stable and not lethal ; it can be used to visualize fungi directly in soil. But it is also used to stain propagules before their introduction into the soil. It enables the growth of the germ-tubes arising from the spores to be followed, at least for a few hours and sometimes for a few days (Tsao, 1970, Scher and Baker, 1983). We used this staining technique to assess the germination rate of conidia and chlamydospores of *F. oxysporum* in soil (Couteaudier and Alabouvette, 1990) and also to demonstrate that strains of nonpathogenic *F. oxysporum* were able to inhibit the germination of the conidia of the pathogen in the rhizosphere of the host-plant (Couteaudier, 1991).
The soil was either sterilized or was not infested with different strains of nonpathogenic *F. oxysporum*. When the inoculum density was stabilized, a suspension of conidia of the pathogenic strain *F. o.* f. sp. *lini* was stained with calcofluor before its incorporation into the soil. The soil was spread as a layer on the bottom of plastic boxes ; flax seedlings were laid on this soil layer and overlaid with another layer of infested soil. After different incubation periods, the roots were carefully removed and each root was shaken in sterile water. This rhizosphere soil suspension was filtered through a Millipore membrane that retained the fungal propagules. The percentage of germinated conidia was determined under a microscope equiped with an epifluorescent illuminator. Comparison of the percentage of germinated conidia with the control ; *ie* germination rate of the pathogen in soil non infested with a nonpathogen, enables to calculate the inhibition rate due to the presence of the nonpathogenic strains. Results showed that some nonpathogenic *F. oxysporum* have a greater ability than others to inhibit the germination of the conidia of the pathogen in the rhizosphere of the host-plant (Couteaudier, 1991).

#### 1.1.2 Use of fluorescein diacetate (F.D.A.)

The fluorescein diacetate is a polar molecule which penetrates into the cells, where it is hydrolyzed by esterases. The hydrolysed molecule accumulates inside the cells which become fluorescent and can be visualized under U.V. light (Babiuk and Paul, 1969). It is possible to quantify the FDA activity in soil and this method has been proposed to assess the microbial biomass of soil (Soderstrom, 1977). As this staining is not specific, it can not

be used in raw soil but it allows to follow the growth pattern and determine the metabolic activity of a given strain of *F. oxysporum* introduced into sterilized soil.
We used this method to compare the heterotrophic activity of two types of inoculum of *F. o.* f. sp. *lini*. Depending on the experiment, we infested the soil either with a suspension of microconidia or with "soil chlamydospore inoculum", *ie* microchlamydospores formed in soil (Couteaudier and Alabouvette, 1990). Having demonstrated that the two types of inoculum demonstrated the same kinetics of survival, we wanted to characterize their respective metabolic activity. The heterotrophic activity was monitored by measuring the rate of hydrolysis of F.D.A. by the method proposed by Schnürer and Rosswall (1972) adapted by Chen *et al.* (1988). Infested soil was added simultaneously with F.D.A. to sodium phosphate buffer. After different periods of incubation, the hydrolysis of F.D.A. was terminated by adding acetone, and the mixture was filtered. The amount of hydrolysed F.D.A. was measured by absorbance at 450 nm with a spectrophotometer and with reference to a standard curve. The specific activity of each inoculum type was determined as the amount of hydrolysed F.D.A. per minute and per colony forming unit. Results showed that for the concentration of $1 \times 10^6$ propagules/g, microconidia did not show any F.D.A. activity, while soil chlamydospore inoculum had an activity of 2.1 g hydrolysed F.D.A./min. These results illustrate the fact that for the same inoculum density different inoculum types may have different metabolic activity. The greater heterotrophic F.D.A. activity of chlamydospores inoculum was correlated with a greater aggressiveness on flax in comparison with conidia inoculum (Couteaudier et Alabouvette, 1990).

### 1.1.3 Comparison of F.D.A. and calcofluor staining techniques

Different staining techniques give different kinds of information. One example illustrates the advantages and limits of 3 different methods used to described the growth pattern of *F. oxysporum* colonizing a sterilized soil. The population dynamics was followed by : - 1 - Soil dilution plate technique to assess the number of colony forming unit on nutritive agar. 2 - Calcofluor staining to quantify and determine the structure of the population (proportion of conidia, mycelium, chlamydospores). 3 - F.D.A. staining to visualize the metabolically active propagules.
Immediately after soil infestation with "talc inoculum" the 3 techniques gave different estimations of the inoculum density : the number of propagules stained by calcofluor was higher than the number of CFU and at the same time there was no F.D.A. activity. This result shows that the microchlamydospores in talc were not metabolically active when introduced into the soil. Some were probably dead because they were stained by calcofluor, but no more able to grow on malt extract. During the growth phase, the population density estimated by soil dilution was similar to the density estimated by FDA staining, while the counts with calcofluor were always higher. On the contrary, after the population reached the plateau, the counts with calcofluor and the numbers of CFU were in good agreement while the F.D.A. activity decreased, clearly indicating that the propagules entered the stationary phase. These results showed that the simultaneous use of the 3 methods enables to accurate analysis of the growth pattern of a fusarium strain in soil.

## 1.2 IMMUNOENZYMATIC STAINING TECHNIQUES

The above described examples showed the need for specific techniques to visualize a given strain of *F. oxysporum* among wild populations of the same fungal species. Even under experimental conditions, in desinfested soil where only two strains are confronted it is absolutely necessary to distinguish between the pathogen and the nonpathogen. The inherent specifity of antibodies suggest that immunoenzymatic techniques would provide such a tool. However, only a few studies have been dedicated to the production of antisera to specifically recognize *Fusarium* species (Clarke *et al.*, 1986). The polyclonal

antibodies obtained are usually able to identify the species *F. oxysporum* but are not able to distinguish between formae speciales and races of *F. oxysporum*. Cross-reactions after exist with related fungi such as *Verticillium* sp. (Gerik *et al.*, 1987). Only the work published by Iannelli *et al.* (1982) indicated the possibility of using antisera to identify some formae speciales within the species *F. oxysporum*. More recently Wong *et al.* (1988) produced a monoclonal antibody able to recognize specifically race 4 of *F. o.* f. sp. *cubense*. But, most of these studies were dedicated to the rapid detection and identification of the specific wilt-pathogen of a given plant but antisera have not been used to study the ecological behaviour of the funGUS in soil or at the root surface. Gerik *et al.* (1987) developed a specific serological staining procedure for *Verticillium dahliae* and used it to study the root colonization of cotton by *V. dahliae* in field soil. Huisman (1982) indicated that this procedure can be used to assess root colonization by *F. oxysporum*.
Following this approach we decided to apply immunoenzymatic coloration technique to study the competitive interaction between a pathogenic and a nonpathogenic *F. oxysporum* at the root surface. All the data related to root colonization by *F. oxysporum* already published (Nagao *et al.*, 1990 ; Mandeel and Baker, 1991) are based on crude isolation techniques that did not allow ar understanding of the phenomena taking place at the root surface. We believe that only an accurate visualization of the initial process of root colonization by pathogenic and nonpathogenic *F. oxysporum* will lead to significant progress.
A soluble protein extract of *F. o.* f. sp. *lini* was used to prepare a rabbit antiserum. The reactivity of this antiserum with the homologous strain was greater than its reactivity to other strains of *F. oxysporum*, however some cross-reactivity was detected with *Verticillium dahliae*. This antiserum was tested against infected plant extracts ; it recognized the infected plant tissues, and gave minimum reaction with healthy plant tissues. Applying the method described by Gerik *et al.* (1987) resulted in a colored precipitate, we have been able to visualize hyphae of *F. o.* f. sp. *lini* at the root surface of the host-plant.
Using the ELISA technique we are now expecting to quantify root colonization by *Fusarium* with this antiserum. As the reactivity of this serum is not specific enough, it will not be possible to use it in raw soil. But we think that it will be usefull to follow the first process of root infection either by a pathogenic or a nonpathogenic strain introduced in a disinfested soil, and to compare the pattern of root colonization by these 2 types of strains. Obviously the next step of this research will be the production of monoclonal antibodies to improve strain specificity.

## 2 - USE OF MARKED STRAINS

To follow the growth or the survival kinetics of a given fungus in raw soil, it is possible to produced marked strains by mutation or transformation. Different techniques such as u.v.. irradiation or chemical mutation can be used to obtain mutants having characteristic morphological features such as an orange colour or mutants being resistant to drugs such as benomyl or antibioticts. Recently, new techniques based on molecular engineering enable the introduction into a fungal strain of bacterial genes that can be used to detect or visualize the fungus in soil or at the root surface. The GUS gene system is the most promising marker for fungi.

### 2.1 USE OF MUTANTS

Mutants of *F. oxysporum* useful for ecological studies are easy to produce. The main problem linked to the use of these mutants is to be sure that the mutation did not affect any important trait of the fungus. Whether the pathogenicity of mutants derived from a pathogenic strain is preserved or not is easy to test, but on the contrary it is difficult to be

sure that a nonpathogenic mutant has the same ecological fitness as the wild strain. Thus, one must be cautious either with the use of such mutants or when discussing the results.
Benomyl resistant mutants have been extensively used to follow the survival kinetics of either pathogenic or nonpathogenic strains in soil. A benomyl resistant mutant of *F. o.* f. sp. *melonis* was used to demonstrate that the suppressiveness of the soil from Châteaurenard was not related to the disappearance of the pathogen that survived as well in the suppressive as in the conducive soil (Alabouvette *et al.*, 1984).
A benomyl resitant strain of *F. o.* f. sp. *lini* was used to assess the competitiveness of different nonpathogenic strains under experimental conditions (Couteaudier and Alabouvette, 1990). A concentration of $1.10^3$ CFU/g soil of 9 nonpathogenic strains was mixed with different concentrations of the pathogenic strain Foln35 in order to obtain several initial ratios of inoculum densities. After 20 to 34 days of incubation the number of propagules of each strain was determined using the soil dilution technique and culturing on both malt agar and malt agar enriched with benomyl to reveal the pathogenic strain. It was therefore possible to determine the population density ratio X/Y at the equilibrum level and to compare it to the initial ration Xo/Yo. The high $r^2$ values of the regression analysis revealed that a linear relationship existed between the ratio of inoculum densities at the plateau (X/Y) and the ratio of inoculum densities incorporated into the sterilized soil (Xo/Yo). The slope of the regression lines varied with the combination of strains. This slope was an indication of strain competitiveness in relation to the pathogenic strain Foln35. This model also enables us to compare the ecological traits of a mutant to the features of the wild type.
Marked mutants have also been used to study the root colonization by pathogenic and nonpathogenic *Fusarium*. Schneider (1984) generated orange mutants by u.v. irradiation and used them to demonstrate that some nonpathogenic *Fusarium* were able to reduce root colonization by the pathogen.

### 2.2 USE OF TRANSFORMED STRAINS

A new approach to mark fungal strains is to introduce by transformation a bacterial gene coding for the production of an enzyme not produced by the wild strain nor by other fungi. Reporter genes have already been used to follow the population dynamics of antagonistic bacteria in soil and in the rhizosphere (Lim *et al.*, 1991) but such studies have not yet been published for fungi. One of the problems was that the use of the well known ßgalactosidase system was limited because many fungal species had this endogenous enzymatic activity. Following the work of Jefferson (Jefferson *et al.*, 1987) who used E.coli *uidA* gene, coding for ßglucuronidase as a reporter gene in higher plants, this GUS system was used to transform fungi and yeasts (Roberts *et al.*, 1989 ; Petering *et al.*, 1991). This enzymatic activity did not exist in fungi nor in higher plants therefore it is possible to visualize specifically the transformed strain which appears blue in the presence of the specific substrate.It is also possible to quantify the glucuronidase activity by fluorimetry, thus we can expect to be able to quantify root colonization by a marked strain of *F. oxysporum* in the presence of wild strains, in raw soil.
The transformation of one strain of *F. o.* f. sp. *lini* was performed by colleagues in Orsay (Couteaudier *et al.*, unpublished). The fitness of the transformed strains was compared to that of the wild strain and we selected a few strains that were still pathogenic and did not show any obvious changes in ecological behaviour, such as growth rate *in vitro* and in soil. Then, we assessed the mitotic stability of the transformed strains. Among the stable strains we chose for further experimentation the strain showing the greater GUS activity.
In preliminary experiments this transformed strain has been used to infect young flax roots. It was possible to visualize the transformed hyphae on the roots and also to quantify root colonization by fluorimetry. Root colonization by the pathogen was reduced in the presence of a nonpathogenic strain.
We believe that we have now got the right tool to study competitive interactions between pathogenic and nonpathogenic *F. oxysporum* in the rhizosphere and in the roots.

## CONCLUSION

In this paper we underlined the need for specific markers to study accurately the competitive interactions between pathogenic and nonpathogenic fusaria in soil. To conclude we would like to insist on another requirement for this type of experimental approach. It is absolutely necessary to take into account the kinetics of the phenomena. Even with specific markers and accurate methods to visualize and quantify the different populations of *F. oxysporum* it is impossible to give a correct interpretation of the data without following the evolution of the populations over time.

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BIOLOGICAL CONTROL OF FUSARIUM WILT OF CARNATION WITH NONPATHOGENIC *FUSARIUM* ISOLATES: EMPHASIS ON COLONIZATION AND INOCULATION METHODS

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Summary
Fusarium wilt in carnation can effectively be suppressed by nonpathogenic *Fusarium* isolates. Suppression with the nonpathogenic *F. oxysporum* isolate 618-12 was only detected when antagonist and pathogen were both applied to the soil, and not if they were inoculated at a different location (stem versus soil). The nonpathogenic isolate 618-12 was present within the stem, sometimes up to 60 cm height, but a distinct active colonization could not be demonstrated.

## Introduction

Fusarium wilt is a soil-borne plant pathogen which causes serious problems in several crops. The difficulties encountered in eradiating Fusarium wilt pathogens by conventional methods, and the fact that adequate resistant cultivars are not always present, make biological control with specific agents an attractive and useful addition to the arsenal of plant disease control measures. Nonpathogenic or less virulent *Fusarium* spp. were found to suppress several formae speciales of *F. oxysporum* in different crops. Also wilt in carnation, caused by *F. oxysporum* f.sp. *dianthi*, can be suppressed by nonpathogenic *Fusarium* strains (Tramier et al., 1987; Cugudda and Garibaldi, 1987; Rattink, 1987; Mattusch, 1990). Favourable aspects of a greenhouse crop for the application of antagonists, are the controlled climatic conditions, and the use of steam-sterilized soil or artificial substrates, which may be easily colonized by the antagonist.
A decrease of 80-100 % of wilted carnation plants can be obtained with nonpathogenic *Fusarium*, however, a rather large variation in the control effect may occur in experiments under similar conditions (Postma and Rattink, 1991). By analysis of the population dynamics of antagonist and pathogen, and by using different inoculation procedures, more information about the mechanism and the optimal conditions can be obtained, which will improve the reliability of biological control.

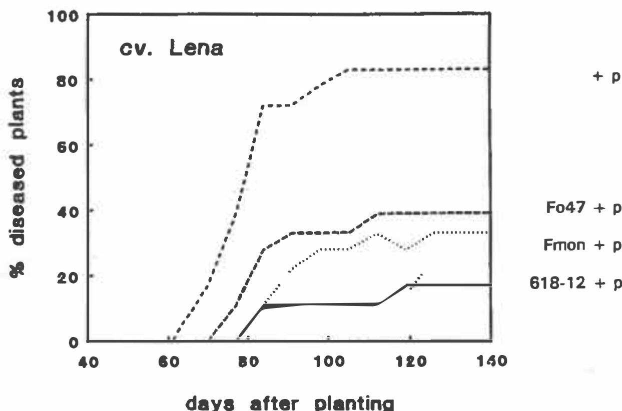
## Experiments

### 1. Comparison of isolates

Rooted cuttings of a susceptible (Lena) and a partly resistant (Pallas) cultivar were grown in steam-sterilized potting soil. All treatments consisted of three replicates with 6 plants grown in separate pots. One, 7 and 20 days after planting, soil was inoculated with 3 ml water, or 3 ml of a suspension containing 4-5 x $10^6$ conidia/ml of the nonpathogenic isolate and at day 26, 3 ml suspension of $10^6$ conidia/ml of the pathogen was pipetted around the stem base. Different nonpathogenic isolates were compared: *F. oxysporum* 618-12, which was isolated from a healthy looking resistant carnation (Rattink, 1987), *F. oxysporum* isolate Fo47 (Alabouvette et al., 1987) and a *F. moniliforme* isolate, Fmon, obtained from R. Tramier (INRA, Antibes). As a control, non-inoculated plants were used.
The three nonpathogenic *Fusarium* isolates significantly ($P < 0.05$) reduced the

number of wilted plants of cultivar Lena (Figure 1). Differences between the three nonpathogenic isolates were not significant. The percentage of diseased plants of the partly resistant cultivar Pallas was not high enough to detect any significant disease suppression.

Figure 1. Disease suppression by pre-inoculation with different nonpathogenic *Fusarium* isolates




2. <u>Different inoculation procedures</u>
To assess the mechanism of the disease suppression by isolate 618-12, stem- or soil-inoculation with 618-12 was followed by stem- or soil-inoculation with the pathogen. Stem- and soil-inoculation with the pathogen alone were used as control treatments.
In this experiment, which lasted for 140 days, a significant disease suppression in cv. Lena was only detected if both 618-12 and pathogen were applicated to the soil. If one was inoculated to the soil and the other was introduced into the stem, no disease suppression was present.

3. <u>Colnization after soil-inoculation</u>
In order to study the colonization of nonpathogenic *Fusarium* in the presence of the pathogen, a benomyl-resistant strain of 618-12 was selected after UV-radiation. This benomyl-resistance was stable, growth and saprophytic ability were similar to the wildtype. Pathogen and nonpathogenic *Fusarium* were inoculated to the soil as described in the first experiment. At the end of the experiment (140 days), soil and carnation stems were sampled. Surface sterilized carnation pieces, cut at 0, 10, 20 ... 70 cm height of the stem, were placed on a *Fusarium* selective medium with or without 10 mg/l benomyl a.i.
The pathogen as well as the benomyl-resistant 618-12, could be reisolated from soil and from the stem. Isolate 618-12 was sometimes present up to 60 cm height. The pathogen was present in all stem pieces of cv. Lena, but it spread less in cv. Pallas. In treatments where both 618-12 and the pathogen were inoculated, plants showed less wilt symptoms, and colonization by the pathogen was less abundant than after inoculation with the pathogen only.

4. <u>Colonization after stem-inoculation</u>
Lena and Pallas were inoculated to the stem 15 cm above the soil with pathogen or 618-12. In order to study the colonization of these isolates, surface sterilized stem pieces of several distances from the inoculation point were placed on *Fusarium* selective medium, 4, 32 and 58 days after inoculation.
The pathogen actively colonized the tissue of Lena and Pallas, but the number

of carnation pieces with 618-12 did not significantly increase during incubation time. To what extent the nonpathogenic isolate is able to colonize carnation, is therefore not clear.

## Discussion

Comparison of three nonpathogenic *Fusarium* isolates showed that isolate 618-12 is at least as effective in the suppression of wilt symptoms in carnation as the other isolates, of which Fo47 was already known to be effective against *F. oxysporum* f.sp. *dianthi* (Tramier et al., 1987; Mattusch, 1990).
The mode of action of this isolate is not yet clear. Several biological control mechanisms have been suggested for the nonpathogenic *Fusarium* isolates: competition in (rhizosphere) soil (Cugudda and Garibaldi, 1987; Tramier et al. 1987; Louvet, 1989), competition for infection sites on the roots (Schneider, 1984), systemic induced resistance, or a combination of these mechanisms (Mandeel and Baker, 1991). Our experiment with carnation, however, did not result in a distinct disease suppression after pre-inoculation with the nonpathogenic isolate at a different location (stem versus soil) than the pathogen. Thus, systemic induced resistance could not be demonstrated. Nevertheless, even after soil-inoculation, isolate 618-12 was present within the carnation stem, sometimes up to 60 cm. Therefore, localized induced resistance or competition between pathogen and antagonist within the vascular system may play a role in the suppression of wilt of carnation in addition to competition in soil and rhizosphere. An active colonization of the stem by isolate 618-12 could not be demonstrated.
More studies on the colonization and experiments with different inoculation procedures are in progress.

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# GLIOCLADIUM CATENULATUM GILMAN ET ABBOTT AS A POTENTIAL BIOCONTROL AGENT TO POTATO TUBER PATHOGENS

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## Summary

*Gliocladium sambucinum* (N 453) was in in vitro experiments, among various other fungi tested, the best antagonist against *Fusarium sambucinum*, the principal agent of potato dry rot in White Russia. Inoculation of wounded potato tubers with a mixed suspension of conidia of *F. sambucinum* and *G. catenulatum* reduced the volume of necrotic tissue 5 times. *G. catenulatum* was in vitro also antagonistic against other potato tuber pathogens such as *Phoma exigua* var. *exigua*, *Geotrichum candidum*, *Alternaria alternata*, and *Fusarium* spp. Microscopical observations revealed different types of hyphal interactions.

## Introduction

Potato is grown as a major source of food in Byelorussia (White Russia). Potato is susceptible to a great number of diseases, about 50 diseases are caused by fungi. Some fungi cause leaf diseases, others cause tuber diseases, and many of them produce symptoms on both plants and tubers. The surface of potato tubers provides a favorable habitat for epiphytic microorganisms, many of them may affect the growth of pathogens. In the light of above, an attempt was made to investigate the possibility of antagonistic interactions of 200 isolates of the genera *Gliocladium*, *Trichoderma*, *Paecillomyces*, *Mortierella*, *Trichocladium*, *Alternaria*, *Scopulariopsis*, *Fusarium*, *Cladosporium*, against *Fusarium sambucinum* Fuckel, the principal causal agent of potato tuber dry rot in Byelorussia.

## Materials and Methods

The fungi isolated from diseased potato tubers just after harvesting or during 1-6 months of storage were tested for their antagonistic activity against *F. sambucinum*. Isolates of possible antagonists were grown on PDA in Petri dishes for 20 days. Spores of 10-day old cultures of *F. sambucinum* were suspended in sterile water ($10^6$ spores/ml) and 2 ml of this was mixed with 20 ml PDA at 60°C. Agar discs (5 mm in diam) with potential antagonist were put in the center of the Petri dishes containing PDA with *F. sambucinum*. The size of the inhibition zone was measured after 5-days incubation.
Isolates most antagonistic to *F. sambucinum* were used for inoculation of potato tubers with mixed conidial suspensions (1:1) of both *F. sambucinum* and possible antagonists (e.g. *Gliocladium*). Spore concentration was $10^6$ spore/ml. Washed, disinfected tubers were damaged up to the 10 mm depth and these holes were filled with the prepared suspension. For every sample 10 tubers were tested. The tubers inoculated with *F. sambucinum* only were used as control. The tubers were placed in a humidity chamber at 23-25°C or in a refrigerator at 5°C. After 10 days the tubers were longitudinally cut through the point of mechanical damage. We measured diameter and depth of

rotted or necrotic area.
The mycoparasitic activity of *G. catenulatum* towards *Geotrichum candidum*, *Verticillium tenerum* was studied in dual cultures. For this purpose two agar discs (2 mm in diam.) with hyphae of either one of the pathogens or the antagonist *G. catenulatum* were placed on the opposite parts of a glass slide, covered with fine layer of PDA. The slides were placed in a humid chamber in Petri dishes at room temperature. The pathogen-antagonist interaction was studied cytologically using light and scanning electron microscopy after 5 days incubation at high humidity.

## Results and Discussion

Byelorussian isolates of *Trichocladium asperum*, *Alternaria alternata*, *Scopulariopsis brevicaulis*, *Stemphyllium* sp., *Fusarium* spp., *Mortierella* spp., and others slightly inhibited the growth of *F. sambucinum*. These inhibition zones varied between 5-8 mm. *G. catenulatum* and *G. roseum* were highly antagonistic, the width of their inhibition zones amounted to 25 and 20 mm respectively (Table 1).

Table 1. Antagonistic activity of *Gliocladium* spp. to *Fusarium* on PDA.

| *Gliocladium* spp. | Isolate number | Zone of inhibition, mm |
|---|---|---|
| *G. catenulatum* | 453 | 25 |
| | 551 | 18 |
| | 585 | 10 |
| | 601 | 16 |
| *G. roseum* | 434 | 15 |
| | 437 | 20 |
| | 474 | 10 |
| | 498 | 20 |
| *G. virens* | 424 | 17 |
| | 456 | 17 |

The antagonistic activity of *G. catenulatum* (isolate 453) to other pathogens of potato tuber rot (*F. avenaceum*, *F. culmorum*, *F. solani*, *F. oxysporum*, *F. graminearum*, *F. lateritium*, *Phoma exigua* var. *exigua* and others) is shown in Table 2.

On potato tubers *G. catenulatum*, isol. 453, was, compared to other isolates, most effective in reducing rot development at both 25°C and 5°C, resulting in a reduction of the necrotic surface of 80% and 89% respectively (Fig. 1). The two other isolates were less effective, particularly at 25°C.

*G. catenulatum* has been reported earlier as a mycoparasite (Simay, 1988), but its mycoparasitic effect on *Fusarium* and other pathogens of potato tubers dry rot (*F. sambucinum*), rubber rot (*Geotrichum candidum*) and wilt disease (*Verticillium tenerum*) has not been reported so far. Our isolate N 453 of *G. catenulatum* caused morphological changes in the tested potato tuber pathogens. As a rule the zone of inhibition was not wide but the growth of the pathogens was inhibited and hyphae were sparse. Hyphae of *G.*

Table 2. The in vitro antagonistic activity of *Gliocladium catenulatum* to different pathogens of potato.

| Test culture | Zone of inhibition (mm) |
|---|---|
| *Fusarium sambucinum* | 25,0 |
| *Fusarium avenaceum* | 18,7 |
| *Fusarium culmorum* | 38,8 |
| *Fusarium solani* | 23,7 |
| *Fusarium solani* var. *argillaceum* | 40,0 |
| *Fusarium oxysporum* | 20,2 |
| *Fusarium graminearum* | 19,5 |
| *Fusarium lateritium* | 21,0 |
| *Phoma exigua* var. *exigua* | 32,0 |
| *Geotrichum candidum* | 21,0 |
| *Alternaria alternata* | 35,0 |
| *Verticillium tenerum* | 21,0 |
| *Trichocladium asperum* | 34,3 |
| *Scopulariopsis brevicaulis* | 20,0 |

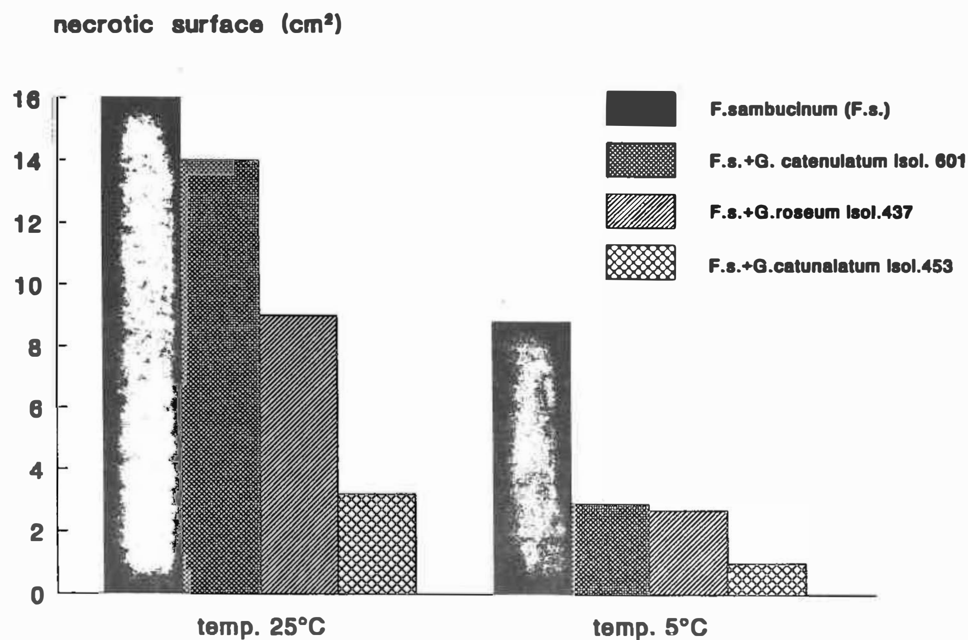


Fig. 1. Effect of *Gliocladium catenulatum* and *Gliocladium roseum* on the development of dry rot of potato tubers at 25°C and 5°C.

*catenulatum* grew over the pathogenic colonies. The types of hyphal interaction commonly were observed coiling and appressed growth. The tight coiling was particularly characteristic for aerial hyphae. *G. catenulatum* formed appressorium-like structures on the hyphae of *V. tenerum*. We found an effect of *G. catenulatum* on the spores of the pathogen such as inhibition of germination, abnormal germ tube growth, destruction of spores, etc. There was variation in the types of hyphal interaction among the species of the pathogens. For example, coiling was not observed in hyphal interaction between *G. catenulatum* and *V. tenerum*. In this case we found the formation of appressorium-like structures. Morphological interactions observed in our experiments were similar to those described for *Rhizoctonia* and *Trichoderma* (Dennis and Webster, 1971), *G. virens* (Tu and Vaarta, 1981). Mycoparasitism of *G. catenulatum* against *F. sambucinum*, *V. tenerum*, *G. candidum*, the principal causal agents of potato root rot in Byelorussia, studied in vitro and in vivo encourages the hope that biological control of potato root rot might become a reality.

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# FOREST SOIL FUNGI FOR BIOCONTROL OF FUSARIUM OXYSPORUM DIANTHI

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## Summary

Forest soils from under old pine stands are considered in Poland suppressive to Fusarium oxysporum, a pathogen causing damping-off of pine seedlings. Fungi isolated from those soils, representing Trichoderma and Penicillium genera, were used for biocontrol of Fusarium oxysporum dianthi. Mixtures of Thrichoderma sp. and Penicillium sp. were applied arround carnation roots and separated at the beginning of the experiment from the peat substratum that contained the pathogen. The treatment resulted in a rate of infection of 4,1 - 20,8% only, while the inoculated control plants were 100% infected after 5 months of experiment.

## Introduction

Suppressiveness of forest podsol soils from old pine stands to Fusarium oxysporum and other fungi causing damping-off of pine seedlings seems to be due to fungi communities inhabiting those soils (Mańka, 1965; Mańka and Kowalski, 1968; Kowalski, 1974). It was supposed that the very same saprophytic soil fungi could also suppress the pathogen of carnation Fusarium oxysporum dianthi (Fod), the main cause of carnation culture failures in Poland. The aim of the work was to find out if some species of forest soil fungi applied in mixtures of two, in a proper way, may be of use for biocontrol of F. oxysporum dianthi.

## Materials and methods

Communities of soil fungi were isolated from podsol soils from under two 80-years-old pine stands in 1986 and 1987, as described by Mańka and Fruzyńska-Jóźwiak (1989). After testing all the isolates for their antagonistic activity to the pathogen in vitro several isolates representing Trichoderma and Penicillium genera were chosen for infection experiments. Inoculum of the antagonistic soil fungi and of the pathogen was prepared on sterile wheat kernels and used for infection experiment on carnation 'Scania 3C' plants grown in Sphagnum peat in plastic tunnel, as described by Manka and Fruzyńska-Jóźwiak (1989). The experiment was carried out from June to October 1990.
There were three controls and eight combinations with soil fungi. The peat control consisted of containers with plants growing in natural peat. In the pathogen control the plants were grown in the substratum mixed with Fod inoculum and in the methodical control carnations were planted into paper bags filled with natural peat and the bags were placed into peat mixed with Fod inoculum. In the other combinations plants were always planted into paper bags containing peat mixed with inoculum of two isolates of forest soil fungi: a Trichoderma and a Penicillium, or two Penicillia in one case. In every combination there were 6 containers with 8 plants in 17 $dm^3$ of substratum.

Plants showing distinct symptoms of Fusarium wilting were collected every week and isolations from their stem bases were made to confirm Fod infection. At the end of the experiment all the plants were harvested and subjected to isolations. The results were subjected to analysis of variance.

## Results and discussion

The results of the experiment are presented in table 1.

Table 1. Appearance of first disease symptoms and infection rate after 23 weeks of experiment

| Combination No | Forest soil fungi species applied together (code) | First sympt. after (weeks) | Infection rate (%) |
|---|---|---|---|
| c | peat control | - | 0[a] |
| pc | pathogen control | 8[a] | 100[b] |
| mc | methodical control | 8[a] | 100[b] |
| 1 | T. harzianum 658 + P. funiculosum 27 | 12[a] | 18,75[c] |
| 2 | T. harzianum 658 + P. nigricans 62/3 | - | 10,4[ac] |
| 3 | T. harzianum 658 + P. nigricans 11 | 18[b] | 20,8[c] |
| 4 | T. viride 79/4 + P. funiculosum 27 | 21[b] | 14,5[c] |
| 5 | T. viride 79/4 + P. nigricans 63/2 | 14[a] | 20,8[c] |
| 6 | T. viride 79/4 + P. nigricans 11 | 13[a] | 14,5[c] |
| 7 | T. viride 85/1 + P. funiculosum 27 | 15[a] | 6,25[ac] |
| 8 | P. adametzi 15/4 + P. funiculosum 27 | - | 4,1[ac] |

a-c - figures followed by the same letters (in columns) do not differ significantly at 0,05

Infection rates in all the combinations with soil fungi differed significantly from those in pathogen and methodical controls, which means that all the mixtures were successful in protecting the plants. The most effective mixtures (no. 8, 7 and 2) did not differ significantly from peat control in which there were no infected plants. Mixtures effective in reducing infection rate were not always successful in delaying the disease development (e.g. No. 7), while the delaying ones resulted sometimes in fairly high infection rate (like No. 3 and 4).

In comparison with results of experiments performed in previous years, a part of which was presented earlier (Mańka and Fruzyńska-Jóźwiak, 1989), it is obvious that the forest soil fungi were able to protect carnations only when they were given priority in contact with plants' roots, before the pathogen. It seems also that there is no difference between the level of protection so that there is no difference between the level of protection by individual isolates and mixtures in 1990 were used separately in 1989 and 1990 with similar results.

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ANTAGONISTIC CHARACTERISTICS OF TRICHODERMA KONINGII TOWARDS VERTICILLIUM DAHLIAE ON PEPPER

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Summary
The investigations were carried out during a 3-years period (1987-1989), using traditional fungicides and a new biocontrol preparation based on the antagonistic fungus *Trichoderma koningii* Oudem. The treatment of the pepper with 0.1% solution of Topsin M (a.i. Thiophanate-methyl) before transplanting in the open and one month after that, lessens the development of the Verticillium wilt with 15-20% in comparison to the control. Best results were received in the variants with the bioproduct Trichodermin, where the degree of the attack by *Verticillium dahliae* is 23-35% lower and the yield is 30-40% higher than those of the control.

## Introduction

Verticillium wilt is an economically important disease on pepper in Bulgaria. The agent was isolated in our country in 1931 from St. Marinov. Elenkov & Hristova (1978) proved, that the disease in Bulgaria is mainly due to *Verticillium dahliae* Kleb. *Capsicum annuum* var. *grossum* and some peppers for milling (variety Gorogled), especially those grown from seedlings are heavily suffering. The specific development of this pathogen in the plant and its accumulation in the soil at more than 30 cm depth makes control difficult. There are no effective chemical means for protection and recovery of the plants. The development of the parasite in the soil is supressed by the soil inhibiting fungi of the genera *Gliocladium* and *Trichoderma* (Papavizas, 1982, 1985; Vajna, 1987). The best investigated antagonists are those, belonging to the genus *Trichoderma* (Seiketov, 1982). Experiments carried out in Bulgaria showed that some strains of *Trichoderma* are highly antagonistic against pathogens of Fusarium wilt on cucumber and carnation (*Fusarium solani f. radicicola, Fusarium oxysporum f. dianthi)*, Pythium rot on cucumber (*Pythium debarianum)* and Phytophthora root rot on pepper (*Phytophthora capsici*). Preliminary studies "in vitro" and in pot trials resulted in three strains of *Trichoderma koningii* Oudem. with a high anatogonistic activity against *V. dahliae* (Georgieva & Georgiev, 1989).

## Material and methods

During the period 1987-1989 field experiments were carried out to establish the efficacy of the experimental bioproduct called Trichodermin against Verticillium wilt on pepper produced for milling ("Gorogled"). The plants were treated with the biocontrol preparation, produced on barley seeds using the selected strain of *T. koningii* N 21 with a titer $2.10^{10}$ spores per gram. The dose was 1 and 2 g per plant. The inoculum of *V. dahliae*, cultivated on barley seeds, was used in a dosage of 15 g on 1 $m^2$ with a titer $2.10^{10}$ spores per gram. The inoculum is incorporated in the soil one day before the transplanting in the field, and Trichodermin was applied during the time of transplanting. Topsin M (a.i. thiophanate-methyl) and Tecto 45 EK (a.i. thiabendazole) were used in concentrations of 0.1% and 0.05% respectively.

The treatment consisted of dipping the roots of the seedlings before transplanting and of pouring 50 ml solution per plant one month after planting. Untreated plants were used as a check.
We estimated the index of Verticillium wilt attack by formula as well as by the McKinney index (Papavizas, 1982).

## Results and discussion

The investigations on the effect of fungicides on the development of Verticillium wilt on pepper showed, that this depends on the frequency of the application. The dipping of the seedlings roots in 0.1% solution of Topsin M lessens the relative degree of attack only with 6% (Fig. 1). The effect increases after the second treatment of the plants by pouring the fungicide solution on to the stem basis one month after transplanting them in the open. Tecto 45 EK (0,05%) shows high a efficacy of 37% wilt reduction, when it is used for dipping of the seedlings, while the twofold treatment resulted in 58% wilt reduction. This fungicide, however, shows phytotoxicity which is expressed in making the plants dwarfed. That is why it falls off as a variant of the trials for the following years. The best results are received in the variants with the application of Trichodermin (Fig. 2).
The efficacy of the treatment with 1 g per plant of Trichodermin is 35% and with 2 g it amounts to 52% which is twice as effective as Topsin M. Consequently, the rate of the antagonistic effect of this preparation to *V. dahliae* has a direct relationship with the dose of application. That is why for the agricultural production the higher dose must be recommended.

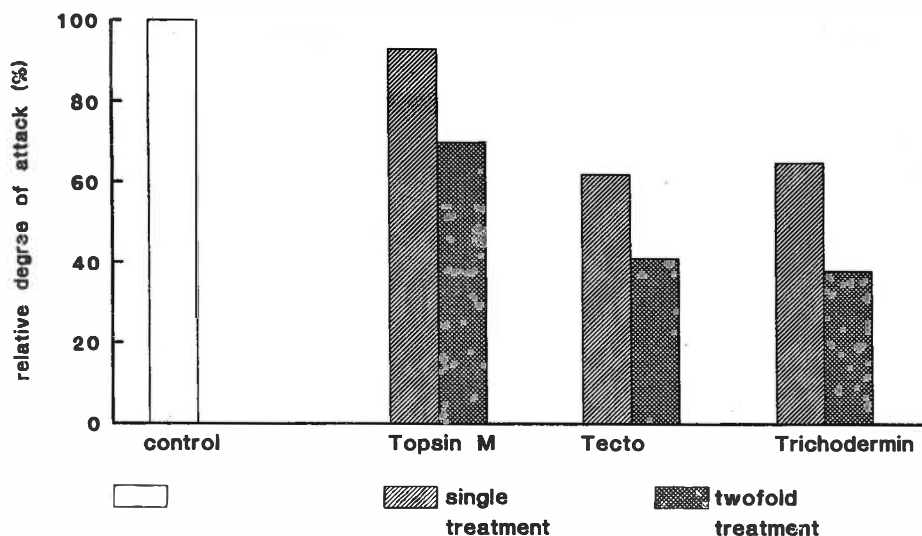


Fig. 1. Development of Verticillium wilt on pepper after fungicide treatment.

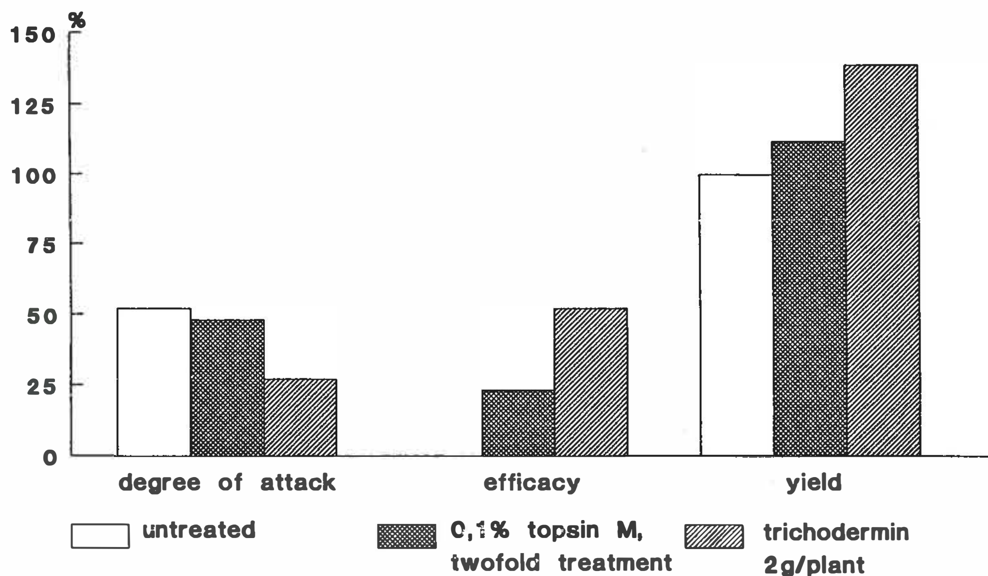


Fig. 2. Effect of Trichodermin and Topsin M against V. dahliae on pepper.

## Conclusions

The strain of *T. koningii* N 21 shows a high antagonistic activity to *V. dahliae* on pepper grown in the field conditions.
The effect of the bioproduct Trichodermin is higher, than that of the systemic fungicide Topsin M.
The advised dosage of the bioproduct is 2 g per plant applied in the soil during the transplanting of the plants in the field.

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*IN VIVO* SCREENING OF POTENTIAL FUNGAL ANTAGONISTS AGAINST *FUSARIUM CULMORUM* IN BARLEY.

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Summary

The antagonistic potential of several saphrophytic fungi against *Fusarium culmorum* on barley seeds was demonstrated.
Different substrates and isolation techniques were used to isolate a number of strains from Danish fields. Some of these have been tested in an *in vivo* screening system using a disease index based on the disease severity caused by two levels of pathogen inoculum.

Introduction.

An internordic project was started in 1989 on the biological control of seed borne diseases of cereals. The objective of the project is to develop screening methods for the selection of antagonists, optimize a seed dressing method and delivery system, and to test the most efficient isolates under field conditions.
In Denmark, activities have been concentrated on the brown foot rot disease of barley caused by *Fusarium culmorum* (W. G. Sm.) Sacc. First, isolation of potential antagonists was carried out. Second, an *in vivo* screening method was used for the selection of antagonists against *Fusarium culmorum* at two inoculum levels.

Methods

In order to increase the likelyhood of finding potential antagonists, isolations were made from soil from an organic and a conventional farm. Sterilized straw, untreated or inoculated with *Fusarium culmorum,* were buried during the winter in the two fields. Isolation was done from samples of soil, straw, rhizosphere-soil from barley seedlings and from germinating barley seeds on wet filter paper.
The standard dilution-plate method, and plating of particles after substrate washing, (Elmholt and Kjøller, 1989) were the main isolation techniques used. 5 different media were employed viz: V8, Dichloran-glycerol agar, *Trichoderma* selective substrate (Elad & Chet, 1983), and soil extract agar or hay infusion agar for soil and straw substrates respectively.
The isolates obtained (app. 800) were stored in 10% glycerol at -80°C. Of these a total of approx. 300 have been tested for antagonism in an *in vivo* screening system developed in Finland (Tahvonen, pers. comm.). Isolates belonging to genera from which antagonistic species have been reported, all isolates of saprophytic *Fusarium* species and some representives of all isolated genera were selected for testing.

In preliminary experiments, inoculum levels of *Fusarium culmorum* of $10^6$ and $10^4$ spores

Tabel 1. Number of isolates of antagonistic fungal species giving 25-100% reduction in the disease index at two inoculum levels ($10^4$ and $10^6$ spores pr ml) of the pathogen *Fusarium culmorum*.

| Antagonistic species | ≥75% reduction | ≥50% reduction | ≥25% reduction |
|---|---|---|---|
| *Acremonium cerealis* | | 2[*2] | |
| *Acremonium* sp. | 1[*1] | 2[*1] | 1 |
| *Aspergillus flavus* | | | 1 |
| *Aurobasidium pullulans* | | | 2[*1] |
| *Cylindrocarpon* sp. | | | 2[*2] |
| *Dendrypion nanum* | 1 | | 1 |
| *Epicoccum purpurascus* | | | 1[*1] |
| *Fusarium dimerum* | | | 2[*2] |
| *F. equiseti* | | | 2[*2] |
| *F. merismoides* | | | 3[*2] |
| *F. oxysporum* (incl. *F. redolens*) | 2[*1] | | 1 |
| *Fusarium sambucinum* | | 1 | |
| *Fusarium tabacinum* | | 1[*1] | 4[*1] |
| *Fusarium poae* | | 1[*1] | |
| *Fusarium* sp. | | | 4[*2] |
| *Gliocladium roseum* | 9[*1] | 2 | |
| *Humicola* sp | 2[*1] | 1 | |
| *Mortierella* sp. | | | 2[*1] |
| *Pencillium hordeii* | | | 1 |
| *Phoma* sp. | | | 3[*2] |
| *Sphaeropsidales* | 1 | | |
| *Trichoderma* sp. | | | 2 |
| *Trichothecium roseum* | | | 1[*1] |
| unidentified | 1[*1] | 1[*1] | 8[*2] |
| Totals | 17 | 11 | 41 |

[*1]: 1 of the isolates was tested in 2 experiments. [*2]: 2 of the isolates were tested in 2 experiments. Nomenclature according to Domsch *et al.* (1980).

per ml were found to cause disease representing the maximum and the minimum level of damage statistical significant in 8 replicates.

The screening procedure was carried out in small serial pots with moistened sand at 15°C. Three seeds were sown in each pot and 8 replicates were made per treatment. Inoculum of the pathogen, *Fusarium culmorum*, was prepared from one subculture and stored in a sufficient number of small ampullae in 10% glycerol at -80°C, giving material enough for all the screening experiments. From 14 days old cultures (PDA) spores were harvested, adjusted to $10^4$ or $10^6$ spores per ml respectively and added in the amount of 1 ml to each 3 seeds. 1 ml of the potential antagonist harvested from 14 days old PDA cultures was added to the seeds immediately after inoculation with the pathogen as a suspension of homogenized mycelia and spores in 0.1% gelatine.
Disease severity was evaluated after 19 days. Disease ratings used were 0= healthy, 1= slightly brown coleoptile and/or roots, 2= moderately brown coleoptile and roots, 3= severe browning of the coleoptile and roots and 4= dead. The disease index was computed as the mean of the characters of the total number of seedlings in one treatment. The reduction in the disease severity was found by comparing the disease index of treated plants with the index of plants to which only the pathogen had been added.

## Results and discussion.

Out of the 300 tested isolates, 69 reduced disease severity caused by *Fusarium culmorum* by more than 25% (tabel 1).
When applied alone, none of the antagonistic isolates were pathogenic on barley.
Isolates belonging to the genera *Gliocladium*, *Trichoderma* and *Fusarium* showed very different levels of antagonism. All tested isolates of *Gliocladium roseum* were antagonistic to some degree, and most reduced disease severity by more than 75% (tabel 1). Only 16 isolates of *Trichoderma* were obtained of which 2 had some antagonistic effect. Saphrophytic *Fusarium* species were very variable in their antagonistic ability. Out of 21 *Fusarium oxysporum* isolates, 3 were antagonistic.
A number of the antagonistic isolates were tested in a second screening, and the results obtained in the first screening were confirmed (tabel 1).
The successful antagonists tested in the screening program have now to be tested in field soil and against other seed borne pathogens e.g. *Bipolaris sorokiniana*. The most effective will be studied further for the mechanisms behind their antagonism and in order to optimize the seed dressing/delivery system.

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# STUDIES ON THE BIOLOGICAL CONTROL OF FUSARIUM CULMORUM IN FINLAND

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## Introduction

In 1989, the Nordic countries made a decision to seek a solution together for the biological control of seed-borne diseases in cereals. Each country was to concentrate on two or three pathogens. The object of study for Finland is the biological control of diseases caused by *Septoria nodorum* and *Fusarium culmorum.* So far we have been working on *Fusarium culmorum* only. In Finland fusarium diseases are the most common root and base diseases among cereals. A fungus highly antagonistic to the pathogen is sought, which could be used for the development of a biological seed disinfectant preparation.

## Methods of investigation

In 1989 and 1990 about 150 field soil samples were collected from various parts of Finland. About 3000 fungus isolates were isolated on dishes, either directly from soil samples or from roots grown in the soil samples. The most common pathogen fungi were excluded beforehand. Isolates were stored at a temperature of -80° C.

In vivo testing was used to distinguish from among the stored isolates the fungi which are antagonistic to *Fusarium culmorum.* The test plant was spring wheat. In the tests that were made indoors the growing temperature was + 15° C.

### 1. Preliminary testing in sand

All the stored isolates were subjected to preliminary testing in sand. Seeds were sown in sand, and mycelium and spore suspension of the pathogen was pipetted on them, followed by mycelium and spore suspension of the test fungus. Suspensions were made from cultures that had been grown on PDA dishes. Finally, the sawn seeds were covered with a thin layer of sand. After a growing period of two and a half weeks the sprouts were collected and the health of roots and bases was evaluated.

### 2. Extended testing in peat

Those isolates that showed antagonistic properties in the sand tests were retested in peat. Because of the lack of seed that is

naturally highly infected by Fusarium culmorum the seed was artificially infected by dipping in suspension made of mycelium and spores of the pathogen. After the surface of the seed had dried, the seed was treated with suspension made of mycelium and spores of the test fungus. After a growing period of two and a half weeks the health of sprouts was evaluated.

3. Tests in field soil
The isolates that were successful in the peat tests were retested in field soil indoors. The treatment of the seed was the same as in the peat test. Results of these tests are not yet available. The isolates that have been shown antagonistic in field soil tests will be tested in field trials at a later date. Field trials on the isolates that were most successful in peat tests have been set up for this summer already.

## Results and discussion

Preliminary testing has been successful. Until now, around 700 isolates have been tested. About 19% of these were antagonistic to Fusarium culmorum (Table 1).

Table 1. Distribution of the most successful isolates in preliminary tests by genus.

| | Number of isolates | | Number of isolates |
|---|---|---|---|
| Trichoderma sp. | 45 | Fusarium redolens | 3 |
| Fusarium sambucinum | 29 | Penicillium sp. | 2 |
| Mortierella sp. | 16 | Alternaria sp. | 2 |
| Fusarium oxysporum | 12 | Cladosporium sp. | 1 |
| Gliocladium sp. | 9 | Fusarium sp. | 1 |
| Gymnoascus sp. | 6 | Scopulariopsis sp. | 1 |
| Fusarium equiseti | 5 | Pythium sp. | 1 |
| Cylindrocarpon sp. | 3 | Paecilomyces sp. | 1 |

Of all the isolates tested in sand about 26% were pathogens, 20% had no effect, and 35% had slightly and 13% clearly reduced pathogenic effect. About 6% of the tested isolates prevented symptoms of disease nearly completely.

Many isolates that showed antagonism in sand test were inefficient in peat tests. Until now, 124 isolates have been tested in peat, and 29 of them maintained their efficiency (Table 2).

Table 2. Distribution of efficient isolates in peat tests by genus.

| | Number of isolates | | Number of isolates |
|---|---|---|---|
| Trichoderma sp. | 12 | Mortierella sp. | 1 |
| Gliocladium sp. | 9 | Cylindrocarpon sp. | 1 |
| Fusarium sambucinum | 3 | Fusarium oxysporum | 1 |
| Fusarium redolens | 2 | | |

It is notable that isolates from the genus Gliocladium were very successful in the tests. A surprisingly large amount of efficient fungi was observed in the genus Fusarium, too. All the efficient isolates were isolated from roots grown in the soil samples. Until now no efficient fungus has been isolated directly from soil samples.

The next step in the study is to continue retesting efficient isolates and, finally, to develop laboratory preparations from the most promising isolates. Testing of stored fungi will be continued.

## METHODS FOR TESTING FUNGAL ANTAGONISTS AGAINST *DRECHSLERA TERES* ON BARLEY SEEDS.

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### Summary
Three methods were compared in an experiment to screen fungal isolates for effect against *Drechslera teres* on barley seeds. Suspension of spores and mycelium mixed with polyvinylalcohol was more effective than autoclaved, reinfected barley seeds as antagonist inoculum. The latter seems to stimulate the pathogen.

### Introduction

*Drechslera teres* is a common pathogen on barley seeds in Norway. The State Seed Testing Station found that 63% of tested seed samples of two-rowed barley cultivated in Southern Norway in 1989 were infected with *D. teres* (mean infection: 2,7%, highest infection: 84%) (Brodal, 1990). As much as 93% of the seed samples of six-rowed barley from the same area were infected by *D. teres* or *Drechslera graminea* (mean infection: 14,9%, highest infection: 88%). *D. teres* is situated as a mycelium in or on the hull and rarely on the seed (Singh, 1962). Seed infection is an important factor for the start of an epidemic in the growing season. Also infected plant debris is important for survival of the pathogen. Primary symptoms of infection on the sprouts from infected seeds is a brown stripe on the coleoptiles or brown spots on the first leaves. A temperature during germination of 3-9°C gave optimal symptom development (Olofsson 1976). To identify antagonists against *D. teres*, fungi were isolated from soil from different part of Norway and also from roots of barley sprouts. I have compared methods for introduction of the potential fungal antagonists in the screening system.

### Materials and methods
Naturally infected seeds of the barley cv. 'Ida' with 96% infection of *D. teres* (determined by the OSMO-method at State Seed Testing Station) were used. Three seeds were planted in sand in small plastic pots (3,2 cm x 3,2 cm x 4,7 cm 'Vefi'-pots in VP-trays), to which water was added to approximately field capacity. The trays were covered with transparent plastic to avoid desiccation and incubated at 10°C in a growth cabinet with 14 hrs light and 10 hrs darkness daily. The experiment was terminated after five weeks and the plants were individually assessed for symptoms of *D. teres*. They were divided into four groups (0= healthy plants, 1= brown coleoptiles, 2= brown coleoptiles and spots on the leaves, 3= dead plants or ungerminated seeds).

Three methods for introduction of potential antagonists were compared for nine fungal isolates. The method of planting one antagonist infected seed in the middle of three *D. teres* infected seeds was compared to planting antagonist infected seed in direct contact with each of the three *D. teres* infected seeds in each pot. In a third treatment *D. teres* infected seeds were treated with a suspension of mycelium and spores of the antagonist.

The potential antagonists were increased on PDA plates (3 plates of each isolate). On two of the plates autoclaved barley seeds were placed on the actively growing cultures for about a week. By this time, antagonists had invaded the seeds which were used as inoculum. In sand filled plastic pots one antagonist infected barley seed was placed in the middle of three D. teres infected seeds, or three antagonist infected seeds were placed in contact with each of the three D. teres infected seeds. The seeds and the antagonists were covered by 1 cm sand. Mycelium and spores from the third plate of each isolate were harvested and suspended in 10 ml of sterile water. Then, 0.5 ml of this suspension was mixed with 0.5 ml of 20 % polyvinylalcohol (PVA). D. teres infected seeds were treated with the seed dressing and three seeds were planted in each pot and covered with a layer of sand.

For all methods of antagonist introduction, untreated D. teres infected seeds were used as control. In the two first treatments autoclaved barley seeds were planted in contact with the seeds infected with the pathogen. Seed treatment with 10 % PVA served as control for the third treatment. Each treatment consisted of 10 pots with altogether 30 plants.

## Results and discussion

The results were recorded five weeks after inoculation and are presented in Table 1. Number of plants which were dead or ungerminated and plants with spots on the leaves, were similar (about 10%) for all the isolates and the different methods for introducing the potential antagonists (data not shown).

When in the contol treatment, the autoclaved seeds were placed in direct contact with each of the three D. teres infected seeds, disease severity increased compared to planting one autoclaved seed in the middle of the pot. This is also the matter for some of the isolates (isol. nr. 554 and 544) tested. For three other isolates (isol. nr. 309, 312 and 313), there were no differences between these two methods. When the antagonists were introduced as spore suspension more healthy plants were produced. Stimulation of the pathogen by nutrients from the autoclaved seeds might explain this result, and it could also be the reason for better disease control with seed treatment than with the two other methods for introduction of the antagonists.

For three of the isolates (isol. nr. 361, 440 and 4181) the method of planting one infected seed in the middle of the pot produced the lowest amount of healthy plants. Three antagonist infected seeds close to the naturally infected seeds gave more healthy plants, and the last method using a seed tratment with a mixture of PVA, spores, and mycelium was best. This is probabely an effect of the amount of the potential antagonists added, or that the organisms were situated at the right place to the right time. The method using a mixture of spores, mycelium and PVA produced a complete cover of the barley seeds with the potential antagonist. By using the two other methods the potential antagonist was less competive with the pathogen because of the distance between the organisms.

Only for one of these isolates tested (isol. nr. 545), the method using a suspension of mycelim and spores together with PVA, gave a significantly lower disease control than the other two methods.

Table 1. Percentage healthy barley plants when nine different fungal isolates were tested for their antagonistic effects. Three different methods were used for introduction of the potential antagonists. The seed lot was 96 % infected by D. teres.

| Isolate nr. | Antagonist inoculum | | |
|---|---|---|---|
| | Autoclaved barley seeds per pot | | Suspension of PVA, spores and mycelium |
| | one seed | three seeds | |
| Control | 17 | 7 | 21 |
| 554 | 23 | 13 | 30 |
| 544 | 50 | 37 | 47 |
| 309 | 20 | 20 | 47 |
| 312 | 23 | 20 | 47 |
| 313 | 37 | 37 | 57 |
| 361 | 30 | 40 | 53 |
| 440 | 30 | 47 | 50 |
| 4181 | 50 | 57 | 70 |
| 545 | 43 | 47 | 33 |

The experiment was performed to identify a reliable method of screening fungi for antagonistic effect against D. teres. The seed treatment with spores and mycelium of the antagonists was more time consuming than the other inoculation methods, but it probabely gave a more correct picture of the antagonistic effect of the isolates.

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POTENTIAL FOR THE BIOLOGICAL CONTROL OF CEREAL EYESPOT DISEASE CAUSED BY *PSEUDOCERCOSPORELLA HERPOTRICHOIDES*

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Summary
Fungi and bacteria isolated from wheat plants and straw were tested for antagonism towards the fungus *Pseudocercosporella herpotrichoides*, the causal agent of cereal eyespot disease, using *in vitro* and *in vivo* methods. A number of known fungal and bacterial antagonists of other soilborne plant pathogens were included for comparison. Several bacteria and fungi showed *in vitro* activity including many of the known antagonists indicating that such tests might detect potentially useful organisms. Isolates of the fungi *Verticillium, Penicillium,* and *Trichoderma* and the bacteria *Pseudomonas* and *Streptomyces* also reduced disease levels *in vivo*. The possible limitations of these tests with respect to the biological control of the pathogen in the field are discussed.

## Introduction
Eyespot caused by the fungus *Pseudocercosporella herpotrichoides (Ph)* is an important disease of temperate cereals. Effective control of the disease was originally achieved with MBC fungicides, but their widespread use quickly led to the development of pathogen resistance in the early eighties (Brown *et al* 1984). The currently recommended compounds such as prochloraz are not always fully effective. The potential for biological control of eyespot using microbial antagonists has not been fully assessed.

## Methods

### Isolation of microorganisms
Bacteria and fungi were isolated from wheat seedlings, plants and straw. The field site used was under continuous wheat cultivation with no record of severe eyespot problems. Growth room grown plants were also used for isolations.

### *Pseudocercosporella* strains
*Ph* exists as wheat (W) and (R) types. As R-types predominate in the field in the UK, an R-type strain was used in all initial experiments. The fungus was grown on malt yeast glucose agar at 19°C and conidia were produced by inoculating tap water agar with suspensions of macerated cultures in sterile distilled water and incubating at 4°C for 4 weeks.

### *In vitro* testing
Bacteria and fungi were tested on different agar media for effects on *Ph*. "Natural" media were made from wheat seedlings and straw. Isolates were inoculated onto plates seeded with *Ph* conidia, incubated at 19°C and interactions observed at 5, 7 and 9 days. Isolates giving rise to inhibition zones were retested. Known antagonists of other soil-borne plant pathogens (see Table 1) were included in the testing so that the ability of the *in vitro* tests to detect potential antagonists could be assessed.

Table 1: Isolates of proven biological control activity

| Isolate identity | Number of isolates | Code |
|---|---|---|
| *Microdochium bolleyi* | 1 | MICRO |
| *Trichoderma harzianum* | 8 | WT, T95, T12, T12B<br>TH1, T4, 1131, 1198 |
| *Fusarium* sp | 2 | C5, C14 |
| *Pseudomonas putida* | 1 | PsPut |
| *Bacillus subtilis* | 8 | BS1-BS8 |
| *Streptomyces griseoviridis* ("Mycostop") | 1 | MYCO |

*In vivo* testing
Potential fungal antagonists were selected on the basis of *in vitro* tests. Autoclaved straw collars were co-inoculated with the test isolate and *Ph* spores, incubated at 19°C for 6 weeks, placed over emerging wheat seedlings (cv. Avalon) and covered with sand. Disease score was assessed according to Scott (1971) after 10 weeks. Potential bacterial antagonists were again selected according to performance *in vitro*. Bacterial suspensions in nutrient broth (NB) were applied three times to wheat seedlings inoculated with *Ph* conidia. Disease score was assessed after 8 weeks.

## Results

*In vitro* testing
The results for the testing on agar are shown in Table 2.

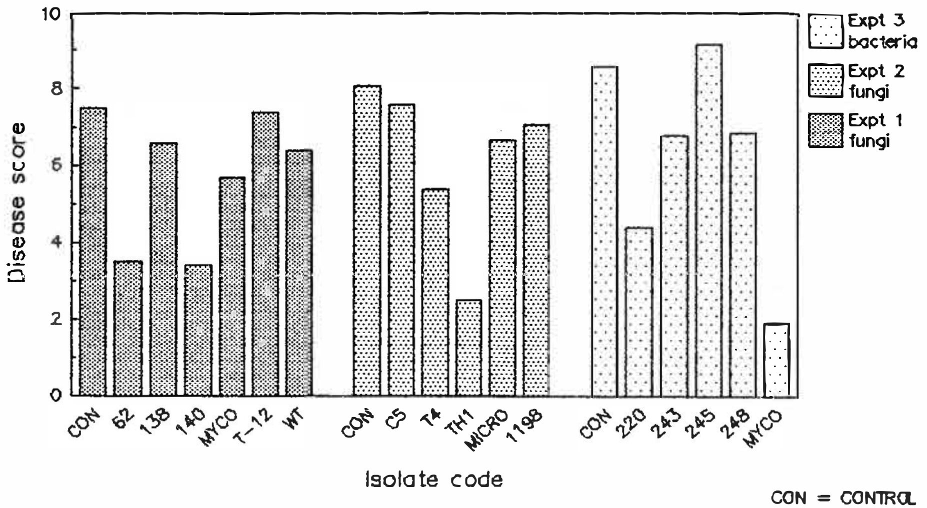
Table 2: Activity of fungal and bacterial isolates against *Ph*

| | | Number of isolates tested | Number showing inhibition zones on NA | PDA | SA | WSA | TWA |
|---|---|---|---|---|---|---|---|
| **Fungi:** | test 1 | 99 | - | 16 | 2 | 2 | 0 |
| | test 2 | 129 | - | - | 1 | 1 | 0 |
| | | | | 18 isolates overgrew plates | | | |
| | known antags | 11 | - | all overgrew plates | | | |
| **Bacteria:** | test 1 | 228 | 23 | - | 5 | 5 | 0 |
| | test 2 | 120 | - | - | 8 | 7 | 2 |
| | known antags | 10 | 8 | - | 8 | 8 | 1 |

NA = nutrient agar
PDA = potato dextrose agar
SA = straw agar
WSA = wheat seedling agar
TWA = tap water agar

*In vivo* testing
The results are shown in Fig 1:

Fig 1: Effect of selected fungi and bacteria on eyespot infection of wheat




## Discussion

### *In vitro* testing

A number of bacteria and fungi were shown to inhibit growth of, or grow over *Ph* colonies on various agar media. Positive results on "natural" or low nutrient media (WSA, SA, TWA) were considered to be the best criteria for selecting potential antagonists to be tested *in vivo*. Positive results were obtained for most of the known bacterial antagonists indicating that the *in vitro* testing method was able to detect potentially useful bacteria. As all the known fungal antagonists and some of the fungal isolates tested overgrew *Ph* colonies, these were all tested *in vivo*.

### *In vivo* testing

Several fungi (*Verticillium* sp. 62, *Penicillium* sp. 140, *Trichoderma harzianum* TH1 and T4) were shown to significantly reduce disease levels. As the fungi were co-inoculated with *Ph* onto straws to infect plants, this does not represent the field situation where *Ph* is the primary colonizer. Hence, experiments are being carried out to assess the performance of these fungi on straws pre-inoculated with *Ph*. Bacterial isolates (*Pseudomonas fluorescens* 220, and *Streptomyces griseoviridis* MYCO) also reduced disease. Further work will aim to increase the efficacy of these strains and determine modes of action of selected biological control agents.

## Acknowledgements

We thank the following for cultures: Prof R Baker (WT, T-95, T-12, T12B, PsPut, C5, C14), Dr S Rossall (BS1-BS8), Dr J Uoti (Mycostop) and Dr J Whipps (TH1, T4).

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# *IN VIVO* SCREENING OF *TRICHODERMA* ISOLATES FOR ANTAGONISM AGAINST *SCLEROTIUM ROLFSII* USING RICE SEEDLINGS.

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Summary

*Trichoderma* isolates from Philippine upland soil were screened for antagonism against a strain of *Sclerotium rolfsii* pathogenic to rice seedlings. A number of *Trichoderma* isolates were selected which showed antagonistic activity to *S. rolfsii*. Rice seedlings treated with both the pathogen and the selected antagonists showed an increase in the seedling height.

The seedling method described in this study is a promising *in vivo* approach for screening isolates of *Trichoderma* for biocontrol activity against *S. rolfsii*. The method is simple, easy to carry out, requires relatively little space and results can be obtained within 14 days. The use of this technique together with suitable *in vitro* assays for high chitinase and ß(1,3) glucanase activity and a test for high saprophytic competitive ability should provide a highly efficient system for the selection of isolates with good biocontrol efficiency.

## Introduction

*Sclerotium rolfsii* Sacc., a soilborne fungus, is considered to be one of the most destructive pathogens as it attacks over 500 species in some 100 plant families. On rice, *S. rolfsii* is an important pathogen causing seedling blight particularly on upland varieties. Whereas several species of *Trichoderma* have been shown to give effective control of *S. rolfsii* in various crops (Papavizas, 1985), there are few reports of *Trichoderma* being utilized to protect rice seedlings against attack by *S. rolfsii*. This paper presents a method in which *Trichoderma* isolates can be screened for antagonism against *S. rolfsii* using rice seedlings.

## Materials and Methods

### 1. Source of fungal isolates and inoculum preparation

*Trichoderma* isolates from Philippine upland soil were grown in a mixture of peat and wheat bran (60 g + 100 g + 230 ml water) while *S. rolfsii*, isolated from infected rice plants was cultured in a mixture of wheat bran: vermiculite: water (1:1:0.5 v/v/v). Incubation of the inocula was done for 10 days at 24-28°C. The pathogen inoculum was air dried, ground and kept in an air tight container at room temperature and used within four months.

### 2. Screening procedure

For the screening test, 1 g of *S. rolfsii* inoculum and 1.5 g of each of the *Trichoderma* isolates (fresh weight) were placed on 2 cm of quartz sand (0.4-0.8 mm) contained in black plastic square pots (7 x 7 x 5.6 cm). The pots were filled with sand and nine seeds of upland rice (cv. UPLRi-7) were sown and watered with liquid fertilizer. Uninoculated pots, pots inoculated with the pathogen alone and pots inoculated with the *Trichoderma* isolates alone served as the controls. All treatments and the controls were replicated four times. The seedlings were grown in a growth chamber at 24-28°C with cycles of 14 hrs light and 10 hrs dark. Seedling height and dry weight were recorded 14 days after sowing and analyzed statistically using a

completely randomized design.

## Results

Using the screening procedures outlined above, a number of isolates of *Trichoderma* from Philippine soil were selected which showed biological control potential against *S. rolfsii*. As can be seen in figure 1, rice seedlings treated with the pathogen and one of the selected antagonists showed an increase in seedling height. Treatment with the pathogen alone caused a significant reduction in plant height while treatment with the antagonists alone gave plant heights which were similar to the uninoculated control. Seedling dry weight measurements, not presented here, gave similar results.

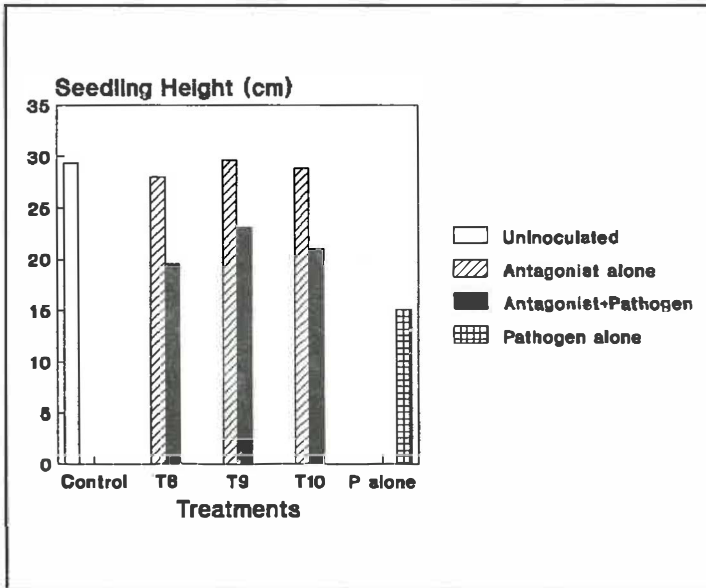


Fig. 1 Height of rice seedlings measured 14 days after treatment with *S. rolfsii* alone (P alone), 3 isolates of *Trichoderma* (T8, T9 and T10) alone, and together with with *S. rolfsii*. An uninoculated treatment served as the control (Control). Data is the mean average of four replicates. Bars with the same letters are not significantly different at LSD 0.05= 3.69.

## Discussion

Screening organisms for antagonism against plant pathogens is a pre-requisite in any biological control study. The initial screening of *Trichoderma* isolates for antagonism is usually performed in the laboratory using *in vitro* methods. After this, extensive testing in the laboratory, greenhouse and field are required. In most cases, attempts to get good correlation between the results from *in vitro* experiments and those of either greenhouse and field studies have failed (Henis *et. al.*, 1984). The reasons for this are discussed further by Baker and Cook (1974). Considering the variability of the pathogen and the environment and the absence of rapid and reliable *in vitro* tests which correlate well with field performance, Chet (1987), recommended the use of test plants in the screening procedure. Baker and colleagues (Henis *et. al.*, 1978; Liu and Baker, 1980 and Chet and Baker, 1981) succeeded in using radish plants

for screening *Trichoderma* spp. against *Rhizoctonia* damping-off disease. Their method is easy and can be carried out within 7-9 days. Elad *et. al.* (1980) used bean seedlings and *Trichoderma* grown on wheat bran. Results from the initial screening correlated well with the results in biocontrol experiments in the field where *Rhizoctonia solani* and *S. rolfsii* were controlled on beans, cotton and tomatoes (Elad *et. al.* 1980). Sivan *et. al.* (1984) utilized cucumber seedlings for screening antagonists against *Pythium aphanidermatum*. The antagonists were grown in a wheat bran/peat mixture. Antagonists selected in the initial screening also provided the same biocontrol activity in tests under greenhouse conditions in which *Trichoderma* spp. were applied either as a wheat bran/peat preparation to the rooting mixture or as a seed coating preparation.

The seedling method described in this study is a useful *in vivo* approach for screening isolates of *Trichoderma* for biocontrol activity against *S. rolfsii*. The method is simple, reproducible, easy to carry out, requires relatively little space and results can be obtained within 14 days. The use of this method together with suitable *in vitro* assays for high chitinase and ß(1,3) glucanase activity (Artigues and Davet, 1984) and a test for high saprophytic competitive ability (Davet, 1986; Davet and Camporota, 1986) might provide an even more efficient system for the selection of isolates with good biocontrol efficiency.

An investigation into the mechanisms of antagonism is in progress and field experiments are to be carried out in the Philippines.

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# TRICHODERMA AS BIOLOGICAL CONTROL AGENT IN SUGAR BEET CROPS

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## Summary

Over the last five years, antagonists were selected and culture collection strains were assayed in order to study the most effective way to control the damping-off of sugar beet seedlings in a natural environment and under greenhouse conditions. *Trichoderma harzianum* was the most effective agent against *Phoma betae* in laboratory and greenhouse conditions. Four strains of *T.harzianum* were selected for their peak efficiency as biological control agents in sugar beet crops.

## Introduction

Fungi of the genus *Trichoderma* are well-known biological control agents (Papavizas, 1985; Davet, 1990). During the last ten years, different strains of *Trichoderma* have been used to control sugar beet seedling damping-off caused by *Rhizoctonia solani, Sclerotium rolfsii* or *Pythium ultimum* and *Aphanomyces* spp. (Lewis & Papavizas, 1987; Camporota et al., 1988; ,etc.).

Since 1986, we have been carrying out studies designed to find antagonist microorganisms of the plant pathogenic fungus *Phoma betae* and of the remaining fungi involved in seedling damping-off and black-leg in sugar beet. Experiments in a controlled environment permitted the selection of four strains of *Trichoderma harzianum* as the most effective biological control agents of this disease. Here, we describe the results obtained in a field of winter sugar beet (December to May) in Córdoba (Spain) sown with seeds coated with the different antagonists selected.

## Materials and methods

1. Pathogens:

Two isolates were used: *P.betae* 10, equivalent to DSM 63181 and CECT 2348, (from sugar beet, Italy) and *P.betae* Bornos (from sugar beet root, Cádiz, Spain). Suspensions of 500,000 conidia/ml were prepared according to Monte & García-Acha (1988).

2. Antagonists:

Four filamentous fungi were selected as biological control agents: *T.harzianum* 2424 (isolated in our laboratory from sugar beet root, Salamanca, Spain), *T.harzianum* IMI 110150 (from Strathclyde, U.K.) and *T.harzianum* B11 and HH3 (kindly donated by Dr.Pierre Davet, Montpellier, France). These strains were grown on potato dextrose agar (PDA) and stored at 4°C for one year. No changes were detected on their antifungal activity.

3. Seed coating:

Monivera monogerm sugar beet seeds with no prior chemical treatment were used. After disinfection by two 10 minute periods of heat treatment at 60°C with an interval of 24h between them, the seeds were coated with acacia gum (2ml/5g of seeds) and activated carbon (6g/5g of seeds). The carbon used to coat the seeds was inoculated with each of the four strains of *T.harzianum.* Five portions of coated seeds were prepared: a control without antagonist, and each antagonist separately. For each 5g portion, 10ml of antagonist suspension obtained by suspending the contents of a 10day PDA plate in 100ml of sterile water was used.

4. Field test:

A field experiment was designed with 5 replicates corresponding to 5 blocks with 15 treatments and 2m passageway as separation between the blocks. The experimental unit was formed by 5m long seed rows, with 400 seeds/row, arranged at 65cm distance between rows. Each row was inoculated at the time of sowing, before the seeds were covered, by spraying with 80ml of a 500,000 conidia/ml suspension of each strain of *P.betae.*

## Results and discussion

From the results shown in Table I, it can be seen that, the infectivity controls having been successful, not only do the antagonists protect the sugar beet from *P.betae,* but also from other pathogens present in the soil, such as *P.betae* itself and *R.solani, R.violacea, Pythium* spp. and *Fusarium* spp. The efficacy of the four strains assayed, with values that in the worst of cases are five times higher than the emergence of control seedlings without antagonist, and the capacity to control the synergic action of the fungi described on beet seedlings in a natural environment can be seen in Table I.
The slight decrease seen starting with the second reading in values undergone by plants emerging from seeds coated with the different isolates of *T.harzianum* was due to the fact they were eaten by birds. However, the relative proportion among the results in the presence of each of the antagonists is maintained and *T.harzianum* 2424 still offers maximum emergency values, with the minimum values corresponding to the coated seed controls and infectivity by *P.betae.* The abnormally high values recorded in the second and third readings with the seeds coated with *T.harzianum* 110150 may have been due to a delay in germination.
2,000 seeds were used for every treatment with an active percentage of germination of 69%.

This study was carried out with support from the projects AGR88-0081 of the Spanish Comisión Interministerial de Ciencia y Tecnología and "Control biológico de hongos" of the Ramón Areces Foundation (Madrid, Spain).

**Table I.** Field experiment (Alameda del Obispo, Córdoba, Spain; December, 1990 - May, 1991). Number of plants emerged from a total of 400 monogerm seeds/5m row sown. The results correspond to the mean value of 5 replicates (2,000 seeds). The percentage of seeds germinated was 69%.

| Treatment | 1st reading 6 weeks | 2nd reading 8 weeks | 3rd reading 10 weeks | 4th reading 12 weeks |
|---|---|---|---|---|
| Control:Coated seeds | 33.80 | 19.80 | 37.40 | 33.80 |
| *T.harzianum* B11 | 261.60 | 221.60 | 239.40 | 227.60 |
| *T.harzianum* 2424 | 274.40 | 251.40 | 239.80 | 214.00 |
| *T.harzianum* HH3 | 244.60 | 238.40 | 228.40 | 205.40 |
| *T.harzianum* 110150 | 159.00 | 179.00 | 196.00 | 170.60 |
| *P.betae* 10 | 30.20 | 25.60 | 26.40 | 23.60 |
| *P.betae* 10 + *T.h.*B11 | 103.40 | 80.20 | 62.00 | 51.20 |
| *P.betae* 10 + *T.h.*2424 | 126.60 | 110.00 | 82.80 | 76.40 |
| *P.betae* 10 + *T.h.*HH3 | 91.20 | 61.00 | 56.00 | 47.60 |
| *P.betae* 10 + *T.h.*110150 | 134.60 | 93.20 | 101.80 | 86.60 |
| *P.betae* Bornos | 45.80 | 41.60 | 42.40 | 35.80 |
| *P.betae* B. + *T.h.*B11 | 158.80 | 115.20 | 114.40 | 96.20 |
| *P.betae* B. + *T.h.*2424 | 131.60 | 87.40 | 92.80 | 83.20 |
| *P.betae* B. + *T.h.*HH3 | 125.60 | 98.80 | 93.00 | 82.60 |
| *P.betae* B. + *T.h.*110150 | 180.60 | 148.40 | 145.60 | 116.60 |
| lsd | 53.94 | 50.30 | 41.30 | 42.43 |

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# BIOLOGICAL CONTROL OF SUGAR BEET DISEASES CAUSED BY *PHOMA BETAE*. GREENHOUSE AND FIELD TESTS.

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## Summary

*Phoma betae (teleomorph:Pleospora betae)* is a seed and soil borne fungus involved in sugar beet diseases such as seedling damping-off, crown and root rot, leaf spots and storage root rot. Two gram-positive endospore forming rods of the genus *Bacillus*, five *Streptomyces-like* actinomycetes, three *Penicillium* isolates and one *Trichoderma* strain were isolated by us from sugar beet plants and soil. The antagonism against different sugar beet pathogenic strains of *Phoma betae* were tested on soil-agar plates in the laboratory. Greenhouse and field experiments showed that eight of these antagonists were able to reduce the infection caused by *P.betae*, and one strain of *Trichoderma harzianum* and one of *Streptomyces* were selected for further studies of biological control.

## Introduction

*Phoma betae* is a phytopathogenic fungus that causes severe injury to sugar beet, table beet and mangold crops, especially in warm regions; damp, rainy climates, and zones susceptible to water-logging. The biological cycle of this fungus was studied by Monte & García-Acha (1988).

The majority of sugar beet diseases produced by fungi have documented chemical, biological and prophylactic treatments. Exceptions to this are those diseases involving *P.betae*, either directly or together with other fungi, such as black-leg or seedling damping-off, crown and root rot, leaf spots and storage root rot (Whitney & Duffus, 1986).

Although *P.betae* can trigger different symptoms during the biological cycle of sugar beet, it is in the seed germination and seedling stages when their multiplication must be controlled, since the development of conidiomata, containing millions of conidia, on the hypocotyl and the root of the seedling, constitutes a source of dissemination of the fungus in the field. In recent years the fungus *Laetisaria arvalis* (Martin et al., 1984) and the bacteria *Bacillus subtilis* (Krezel & Stankiewicz, 1984) and *Pseudomonas* sp. (Blakeman & Brodie, 1977) have been assayed as biological control agents of this pathogen. For the reasons cited above and considering the absence of any effective known treatment of this pathogen in the soil, we have selected and assayed microbial antagonists capable of controlling biologically the diseases produced by *P.betae* on sugar beet crops.

## Results and discussion

1. *Infection by P.betae:*

The symptons of black-leg were reproduced under greenhouse conditions with suspensions of 500,000 conidia/ml from different isolations of *P.betae* at 25,000 conidia/g of soil.

2. Isolation and selection of antagonists:
Two gram-positive endospore-forming rods of the genus Bacillus (C6 and C10); five Streptomyces-like actinomycetes (C1, C2, C4, C7 and C8); three Penicillium strains (C3, C9 and G1); and one Trichoderma strain (2424) -capable of inhibiting the growth of the P.betae colonies- were isolated in corn meal agar. The antagonistic effect of the selected microorganisms on P.betae was assayed on soil-agar plates, by mixing the propagules of the antagonists with soil covered with a fine layer of agar inoculated with a P.betae conidioma. In all cases the plates were incubated at 24 and 28°C for no less than two weeks.

3. Greenhouse experiments:
Multigerm seeds subjected to heat at 60°C for 10 minutes were used, repeating the procedure 24h later. Once cooled, they were coated with 1% water-agar, submerged in an antagonist suspension (10 ml of water per 90 mm plate) and sown in pots with a sterile mixture of peat:sand:silt (2:2:1). After 48h the pots were watered with 10 ml of the P.betae conidial suspension described above. The cultures were kept with controlled light, temperature and humidity for 28 days. The presence of antagonists which interfered with conidial germination and controlled the growth of P.betae favoured the development of beet roots and seedlings. The infection caused by P.betae was curbed when the seeds were protected with spores from different antagonists able to inhibit the pathogen. The antagonists controlled and -in the worst of cases- delayed the appearence of the symptoms of thinning that precede root necrosis and subsequent death of the plant. During the first week of joint development, P.betae was only isolated from roots protected with Bacillus C6 and Streptomyces C7, which showed evident thinning and blackening.
The real struggle between P.betae and its antagonist occurred between days 7 and 21, with thinning and a slight necrosis of the roots occurring alternatively, which the reparing mechanisms of the beet root itself took charge of restoring. After one month, all the cultures protected with antagonists and infected with P.betae developed healthy plants, except for some thinned and necrosed roots, which in the presence of Streptomyces C4 and Bacillus C6 and Streptomyces C8, were 6% and 2% of the total emerged plants respectively.
T.harzianum 2424 was the antagonist offering the best results in the fight against P.betae, controlling the pathogen in concentrations of up to 125,000 conidia/g of soil.

4. Field tests:
The results obtained in two consecutive campaigns (1986-87 and 1987-88) in Córdoba (Spain) are coincident, but with a greater number of emerged plants in the second year (Table I).
The seeds treated with T.harzianum 2424 and Streptomyces C1 offered a percentage of emerged plants higher than the non-inoculated and disinfected control; plants with much lower percentages of symptoms of black-leg than the inoculated control were observed. Both isolates were selected for subsequent studies.

This study was carried out with support from the projects AGR88-0081 of the Spanish Comisión Interministerial de Ciencia y Tecnología and "Control biológico de hongos" of the Ramón Areces Foundation (Madrid, Spain).

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**Table I.** Field tests (Alameda del Obispo, Córdoba, Spain).Percentage of plants emerged and plants with symptoms of black-leg, from seeds treated with 5 antagonists of Phoma betae, corresponding to two consecutive campaigns (1986-87 and 1987-88). Results are the mean value of 5 replicates.

| | % of plants emerged* | | % of plants with symptoms* | |
|---|---|---|---|---|
| Inoculation | 1986-87 | 1987-88 | 1986-87 | 1987-88 |
| C1 | 91.20 | 114.00 | 63.30 | 60.00 |
| *T.harzianum* | 96.60 | 109.70 | 66.67 | 66.67 |
| G1 | 70.46 | 93.67 | 66.60 | 80.00 |
| C6 | 47.20 | 90.33 | 70.00 | 80.00 |
| C8 | 46.20 | 73.33 | 71.67 | 83.30 |
| *P.betae* + C1 | 155.40 | 196.70 | 46.60 | 36.67 |
| *P.betae* +*T.harzianum* | 171.20 | 226.00 | 47.00 | 43.30 |
| *P.betae* + G1 | 128.60 | 194.30 | 47.50 | 46.67 |
| *P.betae* + C6 | 126.00 | 171.70 | 53.33 | 53.33 |
| *P.betae* + C8 | 96.20 | 114.30 | 63.33 | 53.00 |
| *P.betae* | 21.20 | 38.00 | 63.33 | 100.00 |
| Non-inoculated control | 100.00 | 100.00 | 23.45 | 36.00 |
| lsd (0.05) | 51.92 | 9.37 | 39.70 | 12.15 |

* Percentage referred to non-inoculated controls.

## POPULATION STUDIES OF *TRICHODERMA HARZIANUM* AND *PYTHIUM* SPP. AND BIOLOGICAL CONTROL OF DAMPING-OFF AND ROOT ROT OF CUCUMBER IN PEAT FOLLOWING SUBSTRATE AMENDMENT WITH OATMEAL.

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**Summary.**
Cucumber seedlings grown in steamed sphagnum peat amended with various combinations of *Pythium*-infested soil, *T. harzianum* peat-bran inoculum and oatmeal were evaluated for growth, damping-off and root rot.

Damping-off and root rot were markedly reduced in the presence of *T. harzianum*. However, treatments with *T. harzianum* alone had an adverse influence on the growth of the seedlings.

Population studies of the two organisms (measured as cfu on selective media) showed an inverse correlation between the content of *T. harzianum*, the population development of *Pythium* and the disease severity. It seemed, that *T. harzianum* was responsible for an inhibition of the proliferation of *Pythium* thereby reducing the disease severity.

Contrary to *Pythium*, the population development of *T. harzianum* only responded slightly to the oatmeal amendment, suggesting that competition for substrate plays a minor role as a mode of action of *T. harzianum*.

**Introduction.**
The ability of *Trichoderma harzianum* Rifai to control damping-off and root rot caused by soilborne pathogens like *Pythium* spp. has long been known (Papavizas 1985). The present experiment was designed to study the population development of the two organisms relations in the proximity of growing seedlings.

**Materials and methods.**
Peat-bran inoculum of *Trichoderma harzianum* (strain $T_3$) was prepared according to Paulitz *et al.* (1986).

Growth experiments were carried out in steamed peat according to Wolffhechel (1989) and Wolffhechel & Jensen (1991). A soil naturally infested with *Pythium* spp. (mainly *P. ultimum* var. *ultimum*) was used as inoculum (6 g per litre) either without or in combination with oatmeal (3 g per litre). Inoculum of the antagonist was added at the rate of either 0 %, 1 % or 10 % by volume. The ingredients were thoroughly mixed in plastic bags, and the water content adjusted to 22 % (by volume). After 4 days the peat mixture was filled into pots (330 ml each), 3 pots per treatment. The pots were watered (72 % by volume) with a weak nutrient solution. Five 4 day old cucumber seedlings (*Cucumis sativus* L. cv. "Langelands Kæmpe - Gigant") were transplanted into each pot, and placed in a growth chamber at 20°C with a 17 hour photoperiod. The pots were watered to the initial weight on alternate days.

Seven days after planting, the disease severity on roots and collar was rated according to a scale from 0 to 4, where 0 is a totally healthy plant and 4 is a dead plant. The top fresh and dry weights of the plants from each pot were also recorded.

The population development of the antagonist and the pathogen were followed using dilution plating on selective media. For *T. harzianum* TSM (Elad & Chet, 1983) was used, while *Pythium* was isolated on $P_{10}VP$ (Tsao & Ocana, 1969). The first sample was taken the day after mixing and then every second or third day throughout the experiment. Five replicates were made and the results calculated as colony forming units (cfu) per gram dried sphagnum peat.

**Results.**
Damping-off and root rot were markedly reduced in the presence of *T. harzianum*: the greater the amount used the greater the reduction. At 10 % *T. harzianum*-inoculum disease symptoms were reduced to 1/5 of the symptoms developed at the equivalent treatment without *T. harzianum.* However, treatments with *T. harzianum* alone had an adverse influence on the growth of the seedlings, and especially at the highest level of *T. harzianum,* the root system turned brownish yellow and was markedly weakened.

The population of *T. harzianum* was quite constant during the experiment and was only increased slightly following amendment with oatmeal. If *Pythium* was present in the treatments with oatmeal the enhancement failed to appear.

The population density of *Pythium* in treatments without oatmeal never exceeded 100 cfu per gram in combination with 0 % and 1 % *T. harzianum*-inoculum. At 10 % *T. harzianum*-inoculum there was a slight increase to 1,500 cfu per gram. However this was not sufficient to cause disease symptoms. Following amendment with oatmeal the population increased dramatically to 1.7 x $10^5$ cfu per gram (fig. 1). In treatments where *T. harzianum, Pythium* and oatmeal were added together, the population of *Pythium* never exceeded 6.0 x $10^3$ cfu per gram at 1 % *T. harzianum*-inoculum and 28 cfu per gram at 10 % *T. harzianum*-inoculum. Thus, there seems to be an inverse correlation between the content of *T. harzianum*, the population development of *Pythium* and the disease severity, indicating that *T. harzianum* is responsible for an inhibition of the proliferation of *Pythium.*

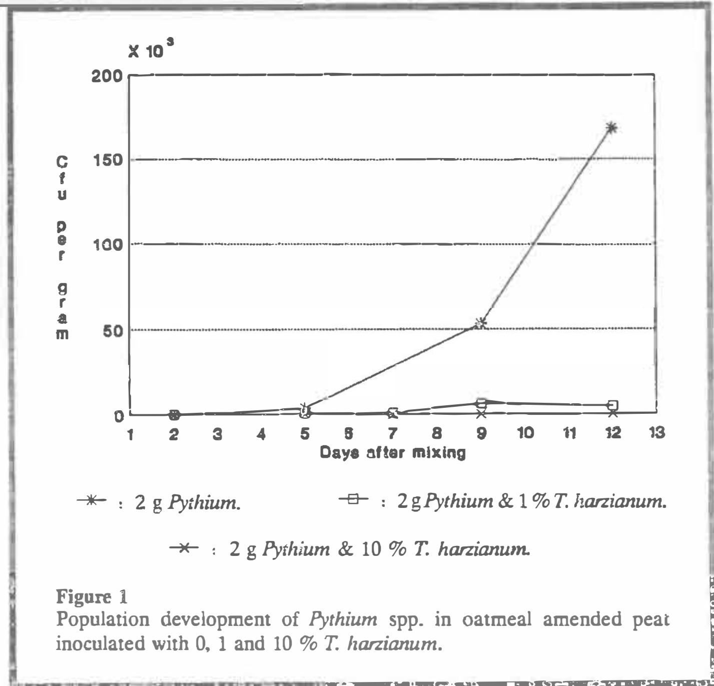

**Figure 1**
Population development of *Pythium* spp. in oatmeal amended peat inoculated with 0, 1 and 10 % *T. harzianum.*

**Discussion.**
Nowadays, special formulations (substrate and carrier media) for the introduction of the antagonist are being used in order to give the antagonist a competitive advantage over other microorganisms including pathogens in the soil.

However, the suggestion has been made, that excess substrate from the formulation used, could stimulate proliferation of major as well as minor pathogens (Kelley 1976), resulting in damage of the plant roots and decreased growth. Results from the present experiment show that neither in the 1 % nor in the 10 % *T. harzianum* peat-bran inoculum treatments did the *Pythium* population densities increase significantly due to the utilization of excess substrate.

Besides, any indigenous minor pathogens will have been eliminated because the *Sphagnum* peat had been steamed prior to use. Thus, when treatments with *T. harzianum* peat-bran inoculum alone had a negative effect on the growth of the seedlings and their root systems, it can only have been caused by *T. harzianum* itself or by unfavorable physical conditions created by the peat-bran formulation. *T. harzianum* has previously been reported to cause damage to root systems of fx maize seedlings (McFadden & Sutton 1975). Contrary to this, most authors have found increased growth of seedlings grown in soil infested with *T. harzianum* (Harman & Taylor 1989, Paulitz *et al.* 1986). The conflicting results could be due to the use of strains with different physiological abilities, or different behaviour of the same strain under diverse edafic conditions. The influence of substrates and the use of "controls", a major problem in this connection, is discussed by Baker *et al.* (1984).

In this study *Pythium* was dependant on substrate amendment with oatmeal in order to increase its population to a level capable of causing detectable disease symptoms. By contrast, the population development of *T. harzianum* only responded slightly to the oatmeal amendment. Thus, because *T. harzianum* caused an inhibition of the proliferation of *Pythium* in treatments with oatmeal, it suggests that competition for substrate plays a minor role as a mode of action. This is in agreement with Lifshits *et al.* (1986) who found, that the addition of nutrients (glucose and asparagine) to seeds coated with *T. harzianum* did not affect successful disease control. It is interesting to speculate whether the production of toxic metabolites by *T. harzianum* strain $T_3$ is more likely to be the principal mechanism of biological control of *Pythium* damping-off.

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**TOWARDS BIOLOGICAL CONTROL OF *PYTHIUM* ROOT ROT IN FORCED TULIPS.**

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## Introduction

Due to restrictions in the use of fungicides other approaches are needed to control *Pythium* root rot in flower production of ornamental bulbs. The development of 'closed' growing systems (de Greeff et al.,1991) in combination with the use of suitable plant substrates may contribute to reduce this problem. Still, additional measures have to be taken when recycling through sterilization is considered. Reintroduction of *Pythium*, making use of the sterilized root debris in the substrate (de Greeff et al., 1991), through irrigation, with soil particles from the surroudings or with contaminated bulbs, might be prevented with antagonists. Screening for antagonists to control root rot in flower production of tulips has recently started by our group in cooperation with R.D.Lumsden (Beltsville, USA). In Baarn, The Netherlands (Weststeijn,1990) a cooperative project (1986-1989) to control *Pythium* root rot in sandy soil with *Pseudomonas* spp. resulted in the selection of a *Pseudomonas* isolate (CBS E11.3). This *Pseudomonas* isolate gave a reduction of root rot in tulips but the level of control varied from experiment to experiment. In this paper preliminary results are presented of experiments performed with five potential antagonists, tested in two different substrates.

## Materials and Methods

*Pythium ultimum* P17, isolated from diseased tulip roots was used as pathogen and the tulip cultivar Gander, prepared as 5°C tulip, was used as host. The pathogen was grown on Czapek Dox agar supplemented with sunflower-oil (2.5 ml/l medium). Four weeks old cultures were homoginized in water and mixed through the substrate giving an inoculum density of $10^6$ cfu per liter substrate. Antagonists used for bulb treatments were applied as suspensions (see also Table 3) from agar cultures; *Pseudomonas* spp.(CBS E11.3) on King's medium B (3 days), *Laetisaria arvalis* (CBS 131.82) on Malt agar (8 days), *Pythium oligandrum* (Bulb Research isolate) on Corn meal agar (8 days), *Trichoderma harzianum* (ATCC 60850) on Potato Dextrose agar (8 days). For substrate treatments, *L.arvalis* and *T.harzianum* were grown on moisted wheat bran (WB) for one week at 20°C and mixed through the substrate to provide 0.5% WB. *Pythium nunn* (ATCC 20693) and *P.oligandrum* were grown on moisted 1% Oatmeal-sand (OS) for three weeks at 20°C and mixed through the substrate to provide 3% OS. *Pseudomonas* spp. was applied to the substrate as a suspension from a 3 days old culture (King's B medium), $10^8$ cfu/ml substraat. Experiments were performed in sandy soil (pH 7, 2% o.m.) and potting mix (pH 6.5). Plant fresh weight and a root rot index were used as parameters to express the severity of *Pythium* disease (Weststeijn,1990).

## Results and discussion

*Effect of plant substrate*

Antagonists applied in potting mix did not show any significant reduction of root rot, nor did it result in any increase in plant weight. This was observed in both bulb and soil treatments (Table 1). In sandy soil, however, significant positive effects were observed when antagonists were applied as

a soil treatment but not as a bulb dip (Table 2). This shows that the effectiveness of a soil treatment depends on the plant substrate used. Accumulation of $CO_2$ in potting mix was more pronounced than in sandy soil, (results not shown) indicating a higher respiration level in potting mix and therefore probably a higher level of microbial activity. This might explain why the introduced antagonists in potting mix are less effective or not effective at all.

*Effect of method of application*

Bulb treatments are less effective in controlling root rot than soil treatments (Table 2). This confirms the results of Weststeijn (1990) who observed a better control of root rot using Pseudomonas spp. as a soil treatment. It might be that the growth rate of the antagonist is too low to keep up with the rather fast growing roots of cold-treated tulips (1 cm/day) resulting in insufficient protection of the roots. When densities of antagonists in suspensions used for bulb dips are considered (Table 3), no effective disease control could be expected from P.oligandrum and L.arvalis. Soil treatments with T. harzianum stimulated root rot as compared to bulb treatments probably because the foodbase used for the soil treatment was not completely colonized by T.harzianum.

*Efficacy of antagonist*

As shown in Table 2, Pseudomonas spp. gave the highest level of disease control in sandy soil when applied to the soil, i.e.,43% increase in plant weight as compared to the inoculated control. Soil applications of P.oligandrum and L.arvalis resulted in resp. 22% and 24% increase of plant weight. L.arvalis applied as a soil treatment (Table 2) without the pathogen resulted in a growth stimulans compared to the control treatment. P.nunn had no or even a negative effect on plant weight. A soil treatment with T.harzianum resulted in a significant reduction of plant weight and an increase in root rot. In potting mix, a soil treatment with T.harzianum also resulted in a reduction of plantweight and in an increase of root rot. The negative effects of P.nunn and T.harzianum might be explained by an incomplete colonization of the food base, which added to inoculated soil, stimulated the pathogen.

*Compatibility with closed growing systems*

The promising results with 3 out of the 5 tested antagonists in the sandy soil treatments encourage us to proceed with these antagonists. Also other antagonists, e.g. Gliocladium virens might be included in these tests. The choice of plant substrate to test these antagonists will depend on the choice made in the development of 'closed' growing systems. We expect that the antagonists will occupy a sterilized, substrate containing root debris, far better than the sandy soil used in these experiments, and, therefore will be more effective in disease control.

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Table 1. Effect of five potential antagonists on Pythium root rot (RRI) and relative plant weight (PW) of forced tulips planted in potting mix.

| antagonist | bulb treatment PW* (RRI)** | soil treatment PW (RRI) |
|---|---|---|
| *control treatments* | | |
| no antagonist | 100 (0.5) | 100 (0.5) |
| Pseudomonas spp. | 106 (0.5) | 94 (0) |
| L.arvalis | 95 (0.7) | 95 (0) |
| P.oligandrum | 107 (0) | 100 (0) |
| T.harzianum | 107 (0) | 61 (2.2) |
| P.nunn | | 94 (0) |
| *Pythium inoculated* | | |
| no antagonist | 74 (4.3) | 63 (4.0) |
| Pseudomonas spp. | 78 (3.7) | 70 (3.0) |
| L.arvalis | 72 (4.5) | 54 (4.5) |
| P.oligandrum | 70 (4.5) | 56 (4.2) |
| T.harzianum | 78 (4.5) | 14 (5.0) |
| P.nunn | | 50 (4.5) |

*Plant weight (PW) is expressed as percentage of the non-inoculated control.
LSD (P=0.05)PW,soil treatment = 8.7; LSD (P=0.05)PW,bulb treatment = 7.7.
** Root rot index; 0=healthy roots, 5=completely rotted root system.

Table 2. Effect of five potential antagonists on Pythium root rot (RRI) and relative plant weight (PW) of forced tulips planted in sandy soil.

| antagonist | bulb treatment PW* (RRI)** | soil treatment PW (RRI) |
|---|---|---|
| *control treatments* | | |
| no antagonist | 100 (0) | 100 (0.2) |
| Pseudomonas spp. | 82 (1.2) | 102 (0.2) |
| L.arvalis | 85 (0.7) | 114 (1.0) |
| P.oligandrum | 88 (0.7) | 113 (0.2) |
| T.harzianum | 94 (0.2) | 103 (0.5) |
| P.nunn | | 99 (0) |
| *Pythium inoculated* | | |
| no antagonist | 63 (3.2) | 69 (3.7) |
| Pseudomonas spp. | 68 (3.5) | 99 (2.0) |
| L.arvalis | 57 (3.8) | 86 (2.5) |
| P.oligandrum | 56 (4.0) | 85 (3.7) |
| T.harzianum | 54 (4.2) | 16 (5.0) |
| P.nunn | | 56 (3.0) |

*Plant weight (PW) is expressed as percentage of the non-inoculated control.
LSD (P=0.05)PW,soil treatment = 14; LSD (P=0.05)PW,bulb treatment = 12.6.
**Root rot index; 0=healthy roots, 5=completely rotted root system.

Table 3 . Densities of antagonists in suspensions used for bulb treatments.

| Antagonist | density (cfu $ml^{-1}$) |
|---|---|
| Pseudomonas spp. | $2x10^9$ |
| L.arvalis | $3x10^3$ |
| T.harzianum | $3x10^6$ |
| P.oligandrum | 10 |

# THE USE OF VESICULAR-ARBUSCULAR MYCORRHIZAL (VAM) FUNGI AS A BIOCONTROL AGENT

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## SUMMARY

The present work covers studies of interaction between two strains of VA mycorrhizal fungi (*Glomus* spp.) and *Pythium ultimum* Trow in cucumber plants (*Cucumis sativus* L.) grown in vermiculite. In all experiments VAM plants were inoculated at sowing. The presence of VAM reduced the incidence of damping-off. This protective effect was found even when the pathogen was introduced simultaneously with VAM. Inoculation with *P. ultimum* 16 days after sowing did not kill the plants, but reduced plant growth. Inoculation with the VAM strains made this effect less profound.

## INTRODUCTION

VAM fungi (*Glomales*) are found in nearly all vegetation systems, including most agroecosystems. However, in horticultural production systems, plants are often grown in media which do not contain a mycorrhizal fungal flora. The potential for the successful introduction of VAM fungi into these substrates must therefore be considered.

Mycorrhizal fungi are involved in nutrient uptake by the plant and this role has been thoroughly investigated. The presence of VAM fungi in root systems has also been found to protect the plant from various stresses including drought, injurious salt concentrations and root diseases caused by bacteria, nematodes and fungi.

The aim of the present experiments was to study the interaction between VAM and the root pathogen *P.ultimum* on cucumber plants.

## MATERIALS AND METHODS

Cucumber (cv. Aminex F.1.) was used as host plant. Two strains of VAM fungi were used: *Glomus etunicatum* Becker & Gerd. (VAM 1) and *Glomus* sp. (VAM 2). Inocula were produced on maize plants grown in calcinated montmorrillonite clay (Terra Green[R]) for two months in a greenhouse, and consisted of spores, mycelia and infected roots. Plants were inoculated with VAM by placing 2 ml of inoculum below the seeds. The *Pythium* inoculum consisted of oospores in vermiculite and dried in Petri dishes at room temperature.

Experiment 1 included the following treatments: a) non-VAM plants ± *P.ultimum* inoculation; b) inoculation with either VAM 1 or VAM 2 at sowing time ± *P.ultimum* inoculation. Cucumber seeds (cv. Aminex F.1.) were sown and grown in speedling trays (75 ml vermiculite/pot; grade 2) and maintained in a glasshouse (20-25°C). Natural light was supplemented by high pressure sodium vapor lamps (30 watt/m$^2$) 24 hours/ day. There were six plants in each treatment, and the plants were inoculated with 4 ml *P.ultimum* inoculum

along the side of the pot at 0, 2, 4, 9 and 11 days after sowing. Control plants received 4 ml vermiculite without *P.ultimum*. Plants were harvested after 20 days and damping-off was recorded. Dry weight (70°C for 24 hr.) of each plant was recorded. The root systems were cleared in 10 % KOH and stained in 0.05 % trypan blue in lactoglycerol. The percentage root length infected with VAM was determined, and the roots were also examined for the presence of *P.ultimum* oospores.

In Experiment 2 seeds of cucumber (cv. Aminex F1) were germinated and two seedlings per pot were planted into 11 cm pots with a mixture of Grodan/vermiculite 1:2 (750 ml/pot). The plants were maintained in a greenhouse (20 -25°C). The treatments that received *P.ultimum* were inoculated 16 days after planting. *P.ultimum* was introduced by applying 20 ml vermiculite inoculum along the side of the pot. Controls received 20 ml vermiculite without *P.ultimum* and the experiment was harvested after 5 weeks. Shoot and root dry weight and % VAM infection was measured.

## RESULTS

In Experiment 1, inoculation with VAM decreased damping-off caused by *P.ultimum*, and the incidence of damping-off declined with increasing plant age before inoculation with *P.ultimum*. Full protective effect by VAM 1 and VAM 2 was found at four and nine days, respectively (Fig.1). The surviving plants showed no significant difference in dry weight or in leaf area compared to the controls. Oospores were found in roots of all plants inoculated with *P.ultimum*, but no attempt was made to quantify them. The root length infected with either of the two VAM isolates did not exceed five percent.

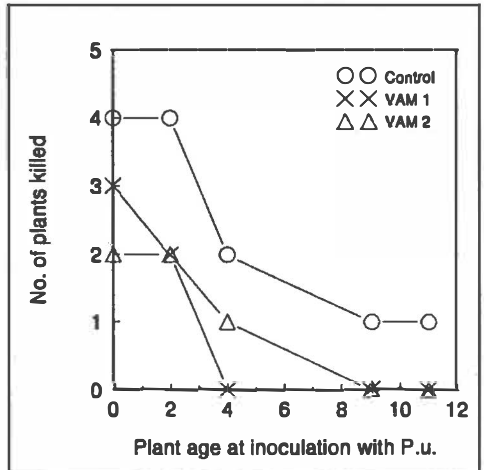


Figure 1. Influence of time of inoculation with *P.ultimum* on the incidence of damping-off of cucumber plants.

In Experiment 2 preinoculation of cucumber plants with VAM fungi diminished the damage caused by *P.ultimum* (Table 1). This was seen as a higher shoot dry weight in plants inoculated with VAM and *P.ultimum* compared to plants that only received *P.ultimum*. Except for a higher root dry weight of plants inoculated with VAM 2, inoculation with VAM did not increase dry weight, when the pathogen was not present. The percentage rootlength infected with VAM was approximately 40 for the two VAM strains.

## DISCUSSION

The protection of VAM against root pathogens has previously been demonstrated, mainly with sub—lethal diseases (Dehne 1982, Rosendahl 1985). In the first experiment (Exp. 1), the protective effect by VAM inoculation was found when the plants were inoculated with

the pathogen simultaneously with VAM inoculation. This protective effect is unexpected, as the VAM infection is not established when the pathogen is introduced. The mechanism involved in the protective effect of VAM is not fully understood. Antifungal metabolites produced in the host plant can not be excluded but are unlikely to play an important role and are being investigated. If host metabolites are involved these could be induced as a response to VAM initiation. However, the pathogen could also be inhibited directly by the VAM fungus by compounds liberated from the VAM spores or mycelium. A more likely explanation is that the presence of VAM reduces the window where plants are susceptible to infection.

In Experiment 2 (Table 1) the VAM fungi were established when *P.ultimum* was introduced. In this experiment the pathogen caused no plant death, but significantly reduced shoot and root dry weight. The reduction was diminished by preinoculation with VAM. This result has previously been found in experiments with VAM-pathogen interactions (Rosendahl 1985, Rosendahl & Rosendahl 1990). The reduced damage of VAM plants is believed to be a consequence of the improved nutrient status of the plant, which enables the plants to surmount the carbon loss caused by the pathogen.

**Table 1. Shoot and root dry weight (g) of plants in Experiment 2 inculated with VAM and *Pythium ultimum* (P.u.).**

| | Shoot dry weight | | Root dry weight | |
|---|---|---|---|---|
| Treatment | 0 | + P.u. | 0 | + P.u. |
| Non VAM | 4.81a | 1.32c | 1.06y | 0.23z |
| VAM 1 | 5.01a | 2.79b | 1.18y | 0.41z |
| VAM 2 | 5.27a | 3.02b | 1.41x | 0.47z |

Values followed by the same letters are not significantly different using Student-Newman-Keuls Test (P>0.05).

The results of the experiments exemplify the potential of VAM fungi as a biocontrol agent. Moreover it is worth noticing that the two VAM strains did not significantly promote plant growth when *P.ultimum* was not present. This illustrates further the relevance of including disease resistance and not only growth enhancement caused by an increase in phosphorus uptake when screening for efficient VAM-isolates for commercial use in greenhouse plants.

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# CONTROL OF PHYTOPHTHORA ROOT ROT OF RASPBERRIES IN NORWAY

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## Summary

Raspberry root rot is a serious disease affecting raspberries in Europe and North America. The pathogen is closely related to *Phytophthora fragariae.* Growing raspberry plants on raised beds decreased disease severity, but did not control the disease completely. Mulching should be avoided in areas were root rot is a problem.

## Introduction

Raspberry root rot has become a serious disease affecting raspberries in Europe and North America. Many species of *Phytophthora* has been recovered from affected plants, but one highly pathogenic species, closely related to *P. fragariae* is responsible for the most major outbreaks (Duncan et al., 1987, Wilcox, 1989). It is suggested to call this species *Phytophthora fragariae* var. *rubi* (unpubl). The other species are only pathogenic when drainage is poor.

The root rot disease causes considerable loss of canes and reduction of yield, and in many cases the complete loss of the affected plantation. All European red raspberry cultivars appear to be susceptible, while some North American cultivars exhibit some resistance. Chemical application with metalaxyl is effective in controlling the disease (Duncan and Kennedy 1989), but as other *Phytophthora* species have developed resistance against metalaxyl, it is nessecary to develope other treatments.

Infection of host roots is by zoospores, which are released in large numbers in free water, and disease development is highly affected by the water condition in the soil. Infected roots produce oospores, which can survive in the soil for many years.

## Material and methods

### Experiment 1

An experiment including different soil treatments was started at Njøs research station in 1990. A split-plot design was used with raised beds (40 cm) compared to flat land as main plots, arranged in three randomized complete blocks, and with two cultivars and five different soil treatments as sub-plots. The sub-plots consist of five plants. The experiment is situated on a river terrasse with a well drained, fine sandy soil containing a small amount of gravel. Trickle irrigation is used for waterering. A root rot infected plantation was removed from the field in 1989, and the field was also
artificial inoculated prior to planting to give a uniform contamination.

### Experiment 2

In spring 1991, a second experiment was planted, using raised beds (0 cm, 15 cm and 30 cm) in combination with a specific race (#41) of *Gliocladium*

virens. The race of Gliocladium virens is from New York State Exp. Station, Geneva, New York, USA. This race (#41) has shown an effect against the pathogenic root rot fungus of raspberry in a pot plant screening test. The experiment is situated close to Experiment 1, with the same soil conditions.

Inoculum was added in individual planting holes. 200 ml inoculum was mixed into 20 l soil giving a rate of 1 cc per 100 cc of soil. Inoculum was prepared by growing the fungus in a mixture of autoclaved peat and bran for two weeks before planting. The plants were also dipped in a slurry of peat-bran inoculum just before planting.

## Results and discussion

### Experiment 1

The results one year after planting show that growing on raised beds decreases root rot attack (table 1). Mulching, however, increases the attacks, and should be avoided when root rot is a problem.

Table 1. Per cent dead plants. Recorded on 1-year old plants.

| | VETEN | | CHILLIWACK | |
|---|---|---|---|---|
| | Flat land | Raised beds | Flat land | Raised beds |
| Grass mulch | 93 | 13 | 27 | 0 |
| Manure mulch | 67 | 0 | 0 | 0 |
| 50% manure in soil | 27 | 7 | 0 | 0 |
| Metalaxyl | 0 | 0 | 0 | 0 |
| Control | 20 | 1 | 0 | 0 |
| **Mean** | **41** | **24** | **5** | **0** |

The effects between flat land and raised beds are most likely to be caused by differences in soil moisture between the treatments. Recording soil moisture by tensiometer in spring 1991, revealed that all sub-plots on raised beds had lower soil moisture than those on flat land, and that mulching gave higher soil moisture than the other soil treatments.

The number of primocanes is higher on raised beds than on flat land (table 2). This effect might be due to a combination of less sporulation and growth of the pathogen as well as higher resistance of the host, because of the lower soil moisture. Perhaps is it also an effect of better growth of raspberry roots independent of pathogen. It is well documented that raspberry plants is very susceptible to water logging, though it is a bit surprising to get this effect in a well drained soil.

On raised beds, sub-plots treated with metalaxyl have developed more primocanes than control plots, indicating that raised beds cannot control the disease completely (table 2). Even plants of the more resistant Chilliwack, seem to be affected by the root rot disease on the raised beds.

Table 2. Number of primocanes per plant. Recorded on 1-year old plants.

| | VETEN | | CHILLIWACK | |
|---|---|---|---|---|
| | Flat land | Raised beds | Flat land | Raised beds |
| Grass mulch | 0.4 | 13.7 | 3.1 | 14.7 |
| Manure mulch | 0.7 | 9.7 | 8.0 | 11.5 |
| 50% manure in soil | 9.8 | 18.1 | 15.8 | 22.5 |
| Metalaxyl | 18.6 | 29.3 | 20.1 | 30.0 |
| Control | 2.1 | 21.3 | 12.6 | 20.2 |
| Mean | 6.3 | 18.4 | 11.9 | 19.8 |

Experiment 2

No root rot symptomes is visible in the experiment with Gliocladium virens, one month after planting.

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CONCEPTS IN MYCOPARASITISM AND BIOLOGICAL CONTROL OF PLANT DISEASES

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## Summary

Events in mycoparasitism are discussed with particular reference to fungi showing potential to act as biological disease control agents. The problems and factors important for developing successful biological disease control programmes in general are described. Reasons for the paucity of commercial biological control agents are considered and possible ways of improving this situation are suggested.

## Introduction

As environmental concerns over the use of chemicals for disease control increase and the number of active ingredients available for use in fungicides decreases, alternative biological methods of disease control have taken on a new importance. The study of mycoparasitic interactions in particular has gained momentum as the antagonistic fungi involved can often have biological disease control potential. This paper is essentially restricted to the concepts associated with mycoparasitism and possible procedures for developing successful biological disease control agents. Many of these aspects have been reviewed in depth recently (e.g. Whipps et al., 1988; Wood & Way, 1988; Lewis et al., 1989; Whipps & Lumsden, 1989, 1991; Baker & Dunn, 1990; Harman & Lumsden, 1990; Hornby, 1990; Baker, 1991; Beemster et al., 1991; Harman, 1991; Lewis & Papavizas, 1991; Whipps, 1991a; Wilson et al., 1991) and readers are referred to these for further detailed information when necessary.

## Mycoparasitism

A mycoparasite may be defined as a fungus growing in intimate association with another from which it derives some or all of its nutrients while conferring no benefit in return. Biotrophic mycoparasites have a persistent contact with or occupation of living cells whereas necrotrophic mycoparasites kill host cells, often in advance of contact and penetration. In the latter case, nutrients are still taken up from the dead host cell.

Mycoparasitism is a commonly observed phenomenon *in vitro* and *in vivo* but with few exceptions the necrotrophs are the major potential biocontrol agents. Some may be ecologically obligate, found actively growing only in association with their hosts in nature. These include the sclerotial mycoparasites *Coniothyrium minitans*, *Sporidesmium sclerotivorum* and *Verticillium biguttatum*, the powdery mildew parasite *Ampelomyces quisqualis* and the rust parasite *Sphaerellopsis (Darluca) filum*. Others are ecologically facultative, including *Pythium oligandrum*, *Gliocladium*, *Talaromyces* and *Trichoderma* species which occur frequently in soil, exhibit a wide host range, and have the ability to attack a variety of fungal structures including hyphae, spores and sclerotia.

A series of events has been observed in mycoparasitic hyphal-hyphal interactions.

Initially location of host mycelium occurs. This is followed, in some cases, by directed growth to the susceptible fungus, presumably in response to release of chemical signals from the host. Recognition, perhaps involving lectins, then takes place and may be followed by coiling or extensive branching of the parasite on the host. Sometimes host degradation may occur before contact or penetration. But commonly, penetration takes place immediately, arising directly from parent hyphae or from appressoria, followed by cytoplasmic breakdown and host exploitation. Depending on the host-parasite combination, cell wall degrading enzymes such as chitinases, $\beta$,1-3 glucanases, proteases and cellulases may be involved in this penetration and exploitation stage as well as locally released antibiotics. Recently, induced release of host lytic enzymes has also been suggested to occur in mycoparasitic Pythium interactions but direct evidence for this is lacking (Laing & Deacon, 1991).

Mycoparasitic hyphal-spore interactions have also been observed. In the phylloplane, ecologically obligate Aphanocladium and Tuberculina species and Sphaerellopsis filum can all attack spores of a wide range of rust genera apparently through production of cell wall degrading enzymes. The less specialized Verticillium lecanii, Scytalidium uredinicola and some Alternaria and Cladosporium species can also attack rust pustules similarly. The yeasts, Stephanoascus flocculosus and S. rugulosus and the Coelomycete Ampelomyces quisqualis can attack both spores as well as mycelium of powdery mildews leading to a reduction of disease and an increase in yield in cucumber (Jarvis & Slingsby, 1977; Jarvis et al., 1989). In the soil, mycoparasitism of long-lived oospores of Aphanomyces, Phytophthora and Pythium species has frequently been reported but there have been no studies of the potential of these fungi to act as biocontrol agents.

In contrast, mycoparasites of sclerotia have shown great biocontrol ability in the glasshouse and field. Of the range examined, perhaps the ecologically obligate Coniothyrium minitans and Sporidesmium sclerotivorum look the most promising although some ecologically facultative Trichoderma isolates have also achieved field control (Backman & Rodriguez-Kabana, 1975; Trutmann & Keane, 1990). As with many hyphal-hyphal interactions, destruction of sclerotia by C. minitans is likely to be facilitated by the release of chitinase and $\beta$,1-3 glucanase by the parasite. This fungus grows slowly, but relatively easily, on a range of media and when applied as a barley-rye-sunflower seed preparation to sunflower seed rows, it reduced Sclerotinia-wilt by 30% over a 2 year period (Huang, 1980). Similarly, when applied as a maizemeal-perlite mixture to soil preplanting, it gave control of Sclerotinia disease of lettuce equivalent to repeated sprays of vinclozolin as long as the disease level was below 40% (Whipps, 1991b). C. minitans survived and spread in soil for over 1 year and continued to degrade sclerotia throughout the period. Apothecial production from sclerotia in C. minitans-treated plots was also reduced.

Sporidesmium sclerotivorum similarly destroys sclerotia in soil but shows some biotrophic features. For instance, haustoria are produced within hyphae of sclerotia of Sclerotinia minor (Bullock et al., 1986) and rather than producing its own $\beta$,1-3 glucanases to degrade sclerotia, the activity of the host enzyme is stimulated (Adams & Ayers, 1983). It is a relatively difficult fungus to grow in large quantities but when applied to soil as a preparation grown on non-sterile sand containing 1% w/v live sclerotia of S. minor, lettuce drop due to S. minor was reduced by 40-80% in 4 successive crops over 2 years (Adams & Ayers, 1982; Adams et al., 1984). Macroconidia of S. sclerotivorum germinated near sclerotia, and after colonization, its hyphae grew through soil from sclerotium to sclerotium, utilizing them as protected nutrient sources (Ayers & Adams,

1981). Significantly, W.R. Grace & Co, USA is in the process of registering *S. sclerotivorum* with the U.S. Environmental Protection Agency for use as a biological disease control agent (G.C. Papavizas, personal communication).

## Planning a disease biocontrol programme

When setting up a disease biocontrol programme a stepwise development strategy is generally adopted. Importantly, the whole process from the method of initial selection to final commercial use must be examined at the outset. Throughout the process the epidemiology and aetiology of the pathogens must be considered along with the crops of interest and their current, and possible future, methods of cultivation.

The first step is to obtain suitable antagonists and this can be carried out in several ways. Most workers try to sample where natural biological control activity is apparent. These environments include suppressive soils where the pathogen is present but no disease occurs or where disease occurred but subsequently died out. The isolation from Chateaurenard soil of a non-pathogenic *Fusarium* strains now undergoing registration for use in France as a biocontrol agent is an excellent example (C. Alabouvette, personal communication). Another strategy particularly relevant to mycoparasites is the baiting of soil with fungal structures. Several good sclerotial mycoparasites have been found in this way and the procedure is in routine use at Littlehampton. A final method, apparently popular with some companies, is to use culture collections, either targeting on specific groups of antagonists of interest or literally anywhere. The latter strategy removes selection bias but resembles searching for a needle in a haystack. With this approach reliance on a screen becomes paramount.

Screens are used to compare the relative activity of new isolates against either known standard biocontrol agents or chemicals in use at the moment against the disease of interest. Commonly, *in vitro* screens are used initially as they are quick, inexpensive and can sometimes indicate possible modes of action. They may also be valuable in indicating the ease of culture of the antagonist and the environmental extremes outside which the antagonist will fail to grow and act. Nevertheless, such screens are totally unrelated to field conditions and those screens based solely on *in vitro* systems rarely produce antagonists active in the field. This is perpetuated to some extent by the funding for biocontrol work being limited frequently to a period of time (2-3 years) too short to allow other screening methods to be developed and applied.

Consequently, the next, or now more frequently, the first, test is an *in vivo* pot test involving the growth of plants, the pathogen and the antagonist in some combination under controlled conditions. This is still fairly quick and easy, and can perhaps indicate field action and as the environment can still be controlled it provides useful ecological data on the antagonist. Nevertheless, it is not a field trial and gives no indication of mode of action. If an antagonist survives to this stage only a field trial remains to be carried out. This too has its pitfalls as there are year-to-year variations in weather, disease development may vary and, if trials are not large enough or suitably replicated, may not take account of localised distribution of the disease. This procedure is also extremely costly and labour intensive explaining why few field trials have been reported.

Concomitantly, with the screening process toxicological information on the antagonist must be obtained and the methods of inoculum production, formulation

and application considered. Related to this is a need for assessment of inoculum quality, shelf life and survival of the antagonist in the field. Throughout this process the cost of production, ease of use and relative efficacy must be compared with existing chemical or cultural measures. If the antagonists are viewed as environmentally friendly, some loss in efficacy or increase in cost may be acceptable but in general they must be seen to match up well with existing control measures to stand a chance in the market. Another important facet is the timing of the decision to apply to the regulatory authorities for an experimental permit and subsequently, for full registration. Commonly there may be delays during these processes and a year's field trials may be lost if the application is not timed appropriately. Links with industry may be valuable for aiding patenting and registration procedures as these activities may be either too costly or complex for individual or small laboratories to undertake. It may also speed up commercialisation. However, such commercial funding may lead to restrictions on freedom to discuss the work at large.

## Problems and future

With the large volume of research now carried out on biological control and new selection and screening methods available it is rather surprising that there are so few commercial biological disease control agents available (see Lewis & Papavizas, 1991 for a recent list). A range of problems can be identified to account for this. They include a lack of reproducibility between results found in the laboratory or the glasshouse and field; cost; lack of efficacy and ease of use in comparison with existing chemical or cultural methods; problems with culture in general or scale up of inoculum production, and clearance for widespread use.

Several approaches are being taken to try to remedy this situation. Having obtained a useful biocontrol strain it may be possible to target screens for more effective isolates of the same organism. Similarly, use of mutagenesis, protoplasting technologies and genetic manipulation may result in improved strains being produced. These methods may also provide strains with increased resistance to agrochemicals and, consequently, allow integration into existing crop management systems at the same time as reducing chemical usage. Several companies are particularly interested in the areas of inoculum production, scale up, formulation and application. They have considerable expertise with fermentation procedures developed through production of pharmaceuticals and food as well as experience with formulation of chemicals for field use. However, in contrast with these procedures yielding metabolites or dead biomass, biocontrol agents produced in fermenters should have both a high yield and be of optimum quality as the biomass must still be viable and active when formulated and applied to the field.

When the improvements described are implemented it is likely that more biological disease control agents will become available. They would have considerable advantages of having been developed using environmentally sound principles and targeted at pathogens in specific ecological niches. Chemical inputs would also be reduced simultaneously. In some cases biological control agents may be self-perpetuating reducing the need for repeated application. This may be ideal for the grower but is not necessarily a property that would be preferred by companies looking for repeated sales of a product. There is also the possibility that resistance to the biocontrol agent would not occur in the target pathogen. Repeated tests with mycoparasites in the laboratory have given no indication of this and presumably ecologically obligate mycoparasites have coevolved with their

hosts. This may be related to the fact that biological control agents may act through several different modes of action simultaneously in contrast to chemicals which generally act by a single mechanism.

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PREVENTION OF WHITE ROT (*SCLEROTINIA SCLEROTIORUM* (LIB.) DE BARY) OF SUNFLOWER AND SOYBEAN BY THE BIOLOGICAL CONTROL AGENT *CONIOTHYRIUM MINITANS* CAMPBELL

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Summary
Results concerning the use of a new biological control agent for Romania - the hyperparasitic fungus *Coniothyrium minitans* - for the prevention of white rot (*Sclerotinia sclerotiorum*) of sunflower and soybean are presented. Under glasshouse conditions the protection of sunflower and soybean plants has been achieved by the application of soil treatments with *Coniothyrium minitans*; the efficiency of these treatments was similar to that of seed treatments using dried biomass of *Trichoderma viride* ($Td_{50}$) and a little lower than that of the specific fungicides used as chemical standards.

## Introduction

References regarding the capability of *Coniothyrium minitans* to parasitize the pathogens: *Sclerotinia sclerotiorum* (Lib.) de Bary, *S. minor* Jagger, *S. trifoliorum* Eriksson, *S. narcissiflora* Gregory, *Botrytis cinerea* Pers. etc. (Şesan, 1989) and the signalization in our country of the hyperparasite (Şesan & Crişan, 1988) have resulted in "in vitro" experiments in order to establish the parameters for the cultivation and multiplication of the fungus (Şesan & Crişan, 1988), followed by "in vivo" experiments to test the capability of the fungus to protect a crop biologically against sclerotinioses.

## Materials and methods

As hosts were used sunflower cultivars and hybrids: HS 82, Florom 305, Record, Fundulea 90, and soybean, Evans cvar. from Fundulea Institute.
The experiments in the greenhouse were conducted at I.C.P.P., Bucharest, and those in the field were carried out at S.E.A, Oradea, Bihor county, during 1988-1989. The experimental variants had 3 replications each and were randomized.
Soil was artificially inoculated with 7-10 days cultures of *Sclerotinia sclerotiorum*, grown on autoclaved grains.
*Coniothyrium minitans* (*C.m.*), grown on PDA, was applied on the soil at a dose of 250 gr/m$^2$.
The effectiveness of the treatment was compared to seed treatment with dried biomass of *Trichoderma viride* Pers. ex S.F. Gray, isolate $Td_{50}$ (2 gr/kg seed), to a standard chemical treatment with a fungicide specific to *Sclerotinia sclerotiorum* (Sumilex 50 WP, Metoben 70, 2 gr/kg seed) and to the untreated check. The data for frequency and yield were statistically analysed by ANOVA.

## Results and discussion

With sunflower, under the glasshouse conditions (Fig. 1), the effectiveness (% reduction of disease) of the biological treatment with *Coniothyrium*

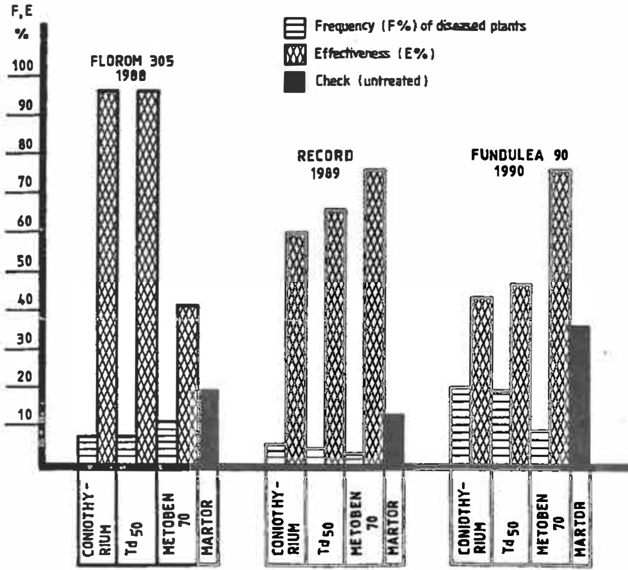


**Figure 1.** Effectiveness of soil biological treatments with ***Coniothyrium minitans*** to prevent ***Sclerotinia sclerotiorum*** on both **sunflower and soybean**, under glasshouse conditions - 1989.

***minitans*** (64.4%) was close to the biological treatment of seed with dry biomass of $Td_{50}$ (67.8%), which demonstrates a control effect (CE) compared with the chemical standard (Sumilex 50 WP) of 90.7% and 95.5%, respectively.
Under field conditions of S.E.A., Oradea (Fig. 2), the effectiveness of soil treatment with *C.m.* was 63-60% in 1988 and 1989, meaning a control effect compared to the standard of 148 and 79%, respectively. During 1990, which was a very dry year, the results were lower and the effectiveness was 43.7% and the CE compared to the standard was 57.5%. Comparing the efficiency of soil treatment with *C.m.* to that of seed treatment with dry biomass of $Td_{50}$, a slightly higher effect of the latter during 1989-1990 and an equal effectiveness of those two types of application in 1988 is to be observed.
In soybean, under glasshouse conditions (Fig. 1), the effectiveness of soil treatment with *C.m.* was only 22.3% while that of the seed treatment with dry biomass of $Td_{50}$ was 30.6%. The effectiveness of the standard Sumilex 50 WP, was also rather low (36.5%) and therefore the CE compared to the standard resulted in rather high values viz. 61.1% for soil treatments with *C.m.* and 84% for seed treatment with $Td_{50}$.
Under field conditions (Fig. 3), the effectiveness of soil treatment with *C.m.* had values which ranged between 86.9% and 63.4%, its control effect during the 3 experimental years, compared to the chemical standard, varied between 120 and 99%. The effectiveness of the seed treatment with $Td_{50}$ dry biomass, varied between 91.7% - 72.7%, with a control effect compared to the chemical standard between 106 and 133%. The effectiveness of soil treatment with *C.m.* was 4.8 - 9.3% lower than that of the seed treatment. Compared to the check with no treatment, both types of treatment with the two hyperparasitic fungi showed good efficiency and the yield increased between 75 and 190 kg/ha. However, during the three experimental years the values of the

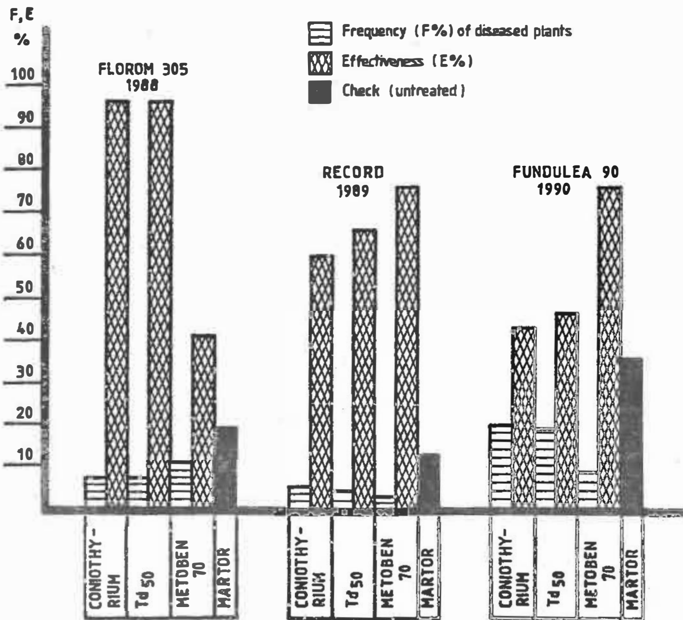


Figure 2. Effectiveness of soil biological treatments with *Coniothyrium minitans* to prevent *Sclerotinia sclerotiorum* on sunflower. Agricultural Research Station Oradea - Bihor county - 1988-1990.

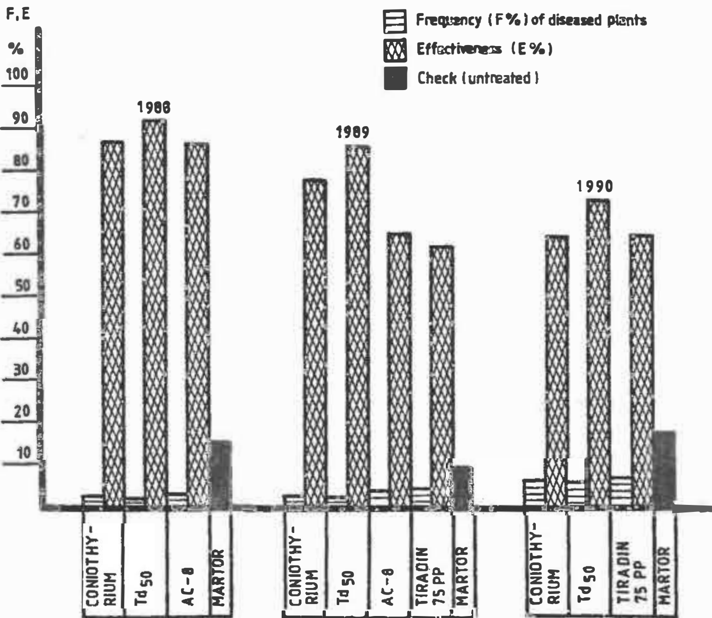


Figure 3. Effectiveness of soil biological treatments with *Coniothyrium minitans* to prevent *Sclerotinia sclerotiorum* on soybean - Evans cvar - Agricultural Research Station Oradea - Bihor county - 1988-1990.

yields were not significantly different.
The results obtained with sunflower regarding protection under the field conditions are the first results of this type of plant protection in our country and are similar to those of Huang (1977, 1980) obtained in Canada and similar to those obtained under glasshouse conditions by Bogdanova & Klimenko (1984).
Our results regarding soya crop protection against white rot cannot be compared to those of other researchers as we could not find such data in literature.
These studies will be continued although the efficiency of biological treatments does not reach the level of those obtained immediately after application of specific fungicides. One must not overlook the possibility of offering an alternative in the frame of the protection technology of both sunflower and soybean to assure the decrease of white rot attack by antagonistic or hyperparasitic fungi. An alternative which can ensure the decrease of the biological resource of sclerotia of *Sclerotinia sclerotiorum* in the soil and at the same time it could avoid the pollution of the ecosystem.

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**VIDEO-ANALYSIS OF GLIOCLADIUM ROSEUM IN RELATION TO MECHANISM OF ANTAGONISM OF PLANT PATHOGENS.**

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Summary

*Gliocladium roseum* was antagonistic towards 10 out of 11 potential hosts screened for hyphal interactions on films of agar. Post-contact antagonism was typified by coagulation of cytoplasm, localised to host hyphal compartments that were contacted. Penetration was never observed. Hyphae of *Pythium aphanidermatum* and *Rhizoctonia solani* were antagonised by pre-contact antibiosis by single hyphae of *G. roseum* at distances up to 350μm and 30μm respectively.

Introduction

A mycoparasite is a fungus which parasitises other fungi. The group encompasses species that are known to secure nutrients directly from their fungal hosts, e.g. *Pythium oligandrum*, and also fungi which antagonise but have not been shown directly to obtain nutrients thereby. To the latter belongs *Gliocladium roseum* and closely related species such as *G.atrum* and *G. catenulatum*. *G roseum* is widespread in British soils. Using a pre-colonised plate method, which selectively isolates mycoparasites, Foley & Deacon (1985) isolated this fungus from 40% of British soil samples. The mechanism of antagonism is uncertain but has been reported to involve coagulation of host cytoplasm as a result of penetration of the host (Walker & Maude, 1978), or production of antibiotic substances (Pachenari & Dix, 1980).

One approach to study of modes of action is by video-microscopy. By recording through thin films of water agar, Laing & Deacon (1991) found that *P. oligandrum* induced rapid post-contact lysis of *Trichoderma aureoviride, Fusarium culmorum* and *Fusarium oxysporum*. Similar techniques were used here to study the mode of action of *G. roseum, G.atrum* and *G. catenulatum* against *F. oxysporum*, and to determine the host range of *G. roseum.*

Results and Discussion

In 10 recorded interactions, *G. roseum* had no pre-contact effect on *F. oxysporum*, but caused a sudden disruption of host cell function at an average 50 min post-contact. On no

occassion was penetration or internal growth of the antagonist observed.Yet narrow hyphae were seen within the damaged host compartments at usually 30 min post-disruption, and on close inspection these were seen to be host hyphae which had regrown from adjacent, undamaged compartments. Within 12 hours of disruption the antagonist proliferated and coiled around the host. *G. atrum* and *G. catenulatum* behaved similarly to *G roseum* in all these respects.

In further, similar studies *G. roseum* was found to antagonise a wide range of fungi. Of 11 fungi screened, 8 were antagonised only after contact. Hyphae of *Pythium aphanidermatum* and *Rhizoctonia solani* were inhibited by pre-contact antibiosis at distances of 350µm and 30µm respectively, and *P. oligandrum* was unaffected by *G. roseum,* but caused rapid post-contact lysis of *Gliocladium.*

Table 1: Fungi found to be susceptible to post-contact antagonism by *Gliocladium roseum.*

| | |
|---|---|
| *Fusarium oxysporum* | *Botrytis cinerea* |
| *Fusarium culmorum* | *Botryotrichum piluliferum* |
| *Trichoderma harzianum* | *Zygorhynchus moelleri* |
| *Trichoderma aureoviride* | *Philophora sp.* (IMI 187786) |

Pachenari & Dix (1980) recorded that when hyphae of *Botrytis allii* encountered those of *G. roseum* their cytoplasm became coagulated and their walls disintegrated. *G. roseum* was suggested to produce a toxin which was effective over a short distance. It is possible that *G. roseum* affects *F. oxysporum* similarly but production of toxins is delayed, or their concentrations are initially so low that only prolonged exposure (mean 50min) causes host disruption. Although coiling of *G. roseum* around *B. allii* hyphae was infrequent (Pachenari & Dix, 1980) it has been reported for *G. catenulatum* on *Fusarium* spp (Huang, 1978), as seen by us in the case of *G. roseum, G. atrum* and *G. catenulatum.* Indeed, the behaviour of these three *Gliocladium* species was indistinguishable in their interactions with *Fusarium oxysporum.*

The extensive host range and prevalence of *G. roseum* (Foley & Deacon, 1985) suggest that it has the potential to be a major regulator of the populations of plant pathogens in soil and in organic substrata. This is reinforced by our findings that it has essentially the same behaviour against poential host fungi on both water agar and nutrient rich agar. There is still no evidence that it benefits directly from its mode of antagonism of other fungi, although its ability to coil round hyphae of them indicates a potential to utilise any materials released from them. Anyhow, its ability locally to inactivate other fungi (post-contact) would enable it to eliminate competition for the underlying substrate, as suggested for *Trichoderma* spp. by Dennis & Webster (1971). *G. roseum* and similar *Gliocladium* species thus seem ideally suited to roles as secondary colonisers of organic residues in soil- the one feature that links all necrotrophic mycoparasites irrespective of their molecular modes of antagonism and that is exploited in the precolonised agar plates used for selective isolation of mycoparasites from soil.

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## STUDIES ON THE INFECTION OF HETERODERA SCHACHTII BY THE NEMATOPHAGOUS FUNGUS VERTICILLIUM CHLAMYDOSPORIUM

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### Summary
Females of the plant parasitic nematode, Heterodera schachtii, are subject to attack by nematophagous fungi such as Verticillium chlamydosporium. Two methods have been used to study this interaction. Large scale pot experiments were conducted in the glasshouse. In addition, plantlets were grown so that their roots could be studied in observation chambers during nematode development. The isolate selected reduced nematode populations significantly. It was rhizosphere competent on oilseed rape and sugar beet. There was no overall difference in the amount of control on these hosts. However, the suppressive effect of the fungus was host dependant. More females were killed on oilseed rape, but a reduction in fecundity attributed to the fungus was more apparent in females from beet. There is therefore an interaction between host, nematode susceptibility and fungal pathogenicity.

### Introduction

Heterodera schachtii is a plant parasitic nematode which attacks sugar beet, oilseed rape and other plants in the Cruciferae and Chenopodiacae. After invasion, females emerge from roots and enlarge to form egg filled cysts. After emerging, females are subject to attack by nematophagous fungi such as Verticillium chlamydosporium. This is a facultative parasite of H.schachtii. Two approaches have been used to investigate infection of this nematode by isolates originally collected from H.schachtii eggs.

### Methods

1. Glasshouse experiments
Beet and rape seedlings were planted in 18cm pots of soil inoculated with a selected isolate of V.chlamydosporium. In some experiments nematode juveniles were added after the plants had established. The subsequent multiplication of fungus and nematodes was determined by quantitative methods. Dilution plating on a selective medium (de Liej et al, 1991) was used to determine colony forming potential of V.chlamydosporium. Cysts were extracted from soil using a fluidising column (Trudgill et al, 1972). Egg infection was assessed by crushing cysts and plating the eggs within onto water agar.

2. Observation experiments
Plantlets were grown in Petri dishes filled with soil. Fungus and nematodes were introduced into soil as in the pot experiments. It was possible to observe development of females on roots, and their subsequent parasitism directly. Numbers of attacked females and their symptoms were recorded. A sample of infected females was tested for egg infection as above.

## Results

In both methods, similar levels of egg infection were obtained with the fungal isolate studied (53%). This isolate is effective in reducing numbers of females by up to 80%.

**Figure 1** Rhizosphere competence in Verticillium chlamydosporium

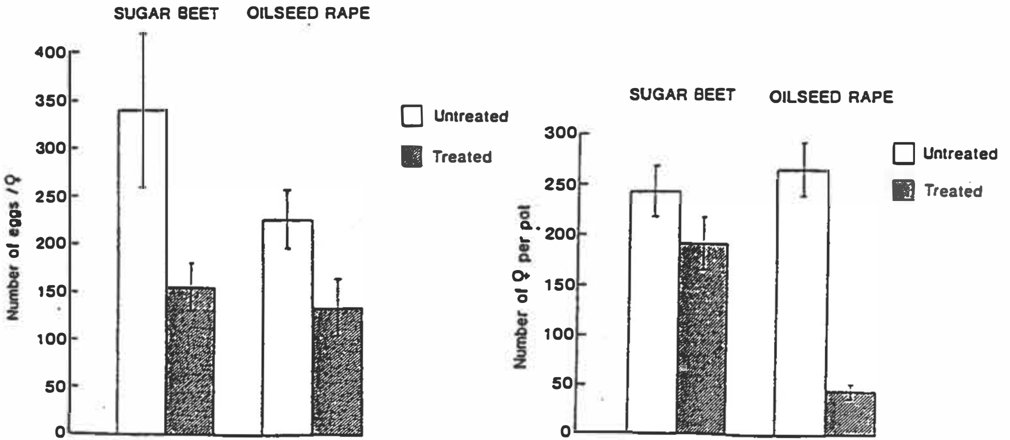


**Figure 2A**

Fecundity of females on beet and oilseed rape treated with Verticillium chlamydosporium compared with untreated controls

**Figure 2B**

Number of females on oilseed rape and sugar beet

Observation experiments revealed that females can take up to 2 weeks to be totally destroyed after showing signs of infection. The first symptom is often a premature tanning of the cyst wall. This is followed by shrivelling. In the later stages some females were surrounded by a halo of chlamydospores. Plating revealed V.chlamydosporium to be present on the roots of all replicates. Not all females became infected although the fungus was growing on the rhizoplane.
Glasshouse experiments showed that this isolate effectively colonised the rhizospheres of beet and rape (fig 1 ). There was no significant difference in counts between the two hosts, although the standard error for beet was consistently greater. Although similar numbers of female nematodes were produced on beet and rape roots, significantly more females were attacked on rape (Fig 2B ). Fig 2A shows that females from beet tended to produce more eggs per individual, and the reduction in fecundity caused by V.chlamydosporium was more pronounced on beet than rape.

## Discussion

The results show that V.chlamydosporium is an effective pathogen and causes a significant reduction of nematode populations on both hosts. It does this by reducing fecundity and killing females.
Rhizosphere competence is important for a fungus to be effective against plant pathogens which invade roots (Ahmad & Baker, 1988). This is in accordance with the results obtained. However, observations showed that the presence of V.chlamydosporium on the roots does not always result in infection of the nematode.
The fungus has different effects on females in beet vs rape rhizospheres. The reduction in fecundity was more obvious for females on beet. These plants have quite different root systems. This may influence fungal growth and so affect pathogenicity. It may also affect nematode susceptibility.
There is no simple relationship between the ability of the plant to support V.chlamydosporium in the rhizosphere and nematode mortality. Statistical tests did not indicate a difference in propagules per gram rhizosphere soil from beet vs rape. However, soil plating does not provide information on the form of the fungus (hyphae, conidia or chlamydospores). The higher standard errors associated with beet colony counts might be expected from the heterogenous morphology of beet roots. It is difficult to evaluate the importance of individual factors in the rhizosphere, such as pH. They may be acting synergistically on the fungus, nematode or both. Further work is in progress to define in more detail factors influencing the suppressive effect of V.chlamydosporium on H.schachtii.

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# SCREENING FOR BACTERIAL ANTAGONISTS AGAINST PYTHIUM WITH BASF PLURONIC POLYOL F-127

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## Summary
BASF pluronic polyol F-127 is a low temperature liquifying polyol which solidifies upon warming. When BASF polyol F127 is used for screening, microorganisms or a target organism are not subjected to a heat shock as when they are embedded in molten agar. Bacteria colonies do not spread when overlayered with BASF pluronic polyol. Broad biological activity against a variety of *Pythium* species was found when bacteria were screened for antagonist activity against *Saprolegnia parasitica*; broad antagonist activity was not seen when *Pythium ultimum*, *P*. type F (Van Der Plaats-Niterink, 1981) or *Botrytis cinerea* were used as targets.

## Introduction

Traditionally, screening for bacterial antagonists has been done with an overlayer technique in which the target organism is embedded in agar. This can be difficult because bacteria colonies spread when covered with molten agar and the bacteria and target organism are subjected to a heat shock when they are embedded in agar. Heat shock can be a problem when bacteria from arctic or high alpine regions are screened and when embedding zoospores or hyphae of some aquatic fungi. The use of BASF pluronic polyol F-127 (BASF Wyandotte, 100 Cherry Hill Road, Parsippany, New Jersey 07054, U.S.A.) a low temperature liquifying polyol which is a block copolymer of polypropylene oxide and ethylene oxide, obviates these problems.

## Materials and Methods

Bacteria which show antagonistic activity against *Pythium ultimum*, *P*. type F (isolate 207) and *Saprolegnia parasitica* were isolated according to the technique of Olson and Lange (1989). The bacteria were grown in liquid nutrient broth cultures on a rotary shaker. The broth, after high speed centrifugation, or a methanol extract of the pellet was then tested against a variety of organisms using a (Biological Activity Spectrum (BAS) test). Inoculum of *Pythium* and *Phytophthora* was produced as follows: the fungi were maintained on sterile grass leaves in sterile water in culture tubes at 4°C; hyphal fragments of these fungi were produced by brief vortex mixing of the culture. Hyphal fragments or spores of the target fungi were used to inoculate liquified nutrient BASF-F127 medium. In the BAS test, 30 µl aliquots of the centrifuged culture broth or methanol extract was applied to Whatman AA filter discs (6 mm diam). The inhibition zone around the filter disc was scored as follows: 8 mm diam = 1/2, 10 mm diam = 1, 15 mm diam = 2, 20 mm diam = 3, 25 mm diam = 4 and 30 mm diam = 5.

Table 1. BAS test (isolates selected for anti Pythium activity)

| Isolate | anti Sp | P207 | P12 | Pu | Pt | Pa | Pi | Po | Ph186 | Ph360 | B | A | R | F | C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 594 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 595 | 0 | 4 | 0 | ½ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 596 | 0 | 4 | 0 | ½ | 0 | 0 | 0 | ½ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 597 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 605 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 606 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 607 | 0 | ½ | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 608 | 0 | ½ | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 609 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | ½ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Sp = Saprolegnia parasitica
P207 = Pythium sp. 207 (F type)
P12 = Pythium sp. 12 (F type)
Pu = Pythium ultimum
Pt = Pythium torulosum
Pa = Pythium aphanidermatum
Pi = Pythium irregulare
Po = Pythium oligandrum
Ph186 = Phytophthora sp. 186
Ph360 = Phytophthora sp. 360
B = Botrytis cinerea
A = Alternaria brassicae
R = Rhizoctonia solani
F = Fusarium oxysporum
C = Colletotrichum dermatium

Zone of inhibition – Use Whatman AA filter disc, 6 mm diam. Use 30 μl/filter disc. ½ = 8 mm diam; 1 = 10 mm diam; 2 = 15 mm diam; 3 = 20 mm diam; 4 = 25 mm diam; 5 = 30 mm diam.

Table 2. BAS test (isolates selected for anti Saprolegnia activity)

| Isolate | anti Sp | P207 | P12 | Pu | Pt | Pa | Pi | Po | Ph186 | Ph360 | B | A | R | F | C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 829 | 1 | 0 | 0 | 0 | 0 | ½ | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 832 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 |
| 833 | 2 | 2 | 0 | 1 | 0 | ½ | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 |
| 836 | 3 | 0 | ½ | 2 | 0 | ½ | 2 | 0 | 0 | 0 | 0 | 4 | 1 | 0 | 0 |
| 837 | 2 | 3 | 0 | 1 | 0 | ½ | 3 | 0 | 2 | 0 | 1 | 4 | 3 | 0 | 0 |
| 839 | 2 | 1 | 0 | ½ | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| 840 | 2 | 1 | 1 | ½ | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 3 | 0 | 0 |
| 841 | 2 | 1 | 0 | ½ | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 4 | 1 | 0 | 0 |
| 868 | 2 | 0 | 1 | 0 | 2 | 1 | 2 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 869 | 1 | 1 | 3 | 0 | 3 | 3 | 3 | 3 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| 870 | 1 | 0 | 3 | 0 | 2 | 2 | 3 | 3 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 871 | ½ | 0 | 0 | ½ | 2 | 3 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 873 | 3 | 2 | 3 | 2 | 3 | 3 | 3 | 3 | 1 | 2 | 0 | 0 | 0 | 0 | 0 |
| 874 | 2 | 0 | 3 | ½ | 2 | 3 | 3 | 3 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| 875 | 3 | 2 | 0 | ½ | 2 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 879 | 2 | 0 | ½ | 0 | 1 | ½ | 2 | 1 | 2 | 0 | 0 | 3 | 3 | 0 | 0 |
| 884 | ½ | 2 | 0 | 1 | 3 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 885 | ½ | 2 | 1 | 2 | 3 | 2 | 2 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 886 | ½ | 3 | 0 | ½ | 3 | 2 | 2 | 0 | 4 | ½ | 0 | 0 | 0 | 0 | 0 |
| 887 | 1 | 0 | 0 | 0 | 1 | ½ | ½ | 0 | 0 | ½ | 0 | 3 | 4 | 0 | 0 |

For figure legend see table 1.

## Results

In table 1 is shown the BAS test results for bacterial antagonists isolated for activity against Pythium ultimum or P. Type F (isolate 207) (Van Der Plaats-Niterink, 1981) and in table 2 the bacteria were isolated for activity against Saprolegnia parasitica. The horizontal lines in the tables indicate that the bacteria were isolated from different ecological habitats.
The results show that isolates selected for antagonistic activity against Pythium (table 1) do not have a broad biological activity spectrum; in fact, these isolates appear to be quite specific in their antagonist activity. (Isolates 593-597 were selected for anti P. type F activity while isolates 605-609 were selected for anti P. ultimum activity). Some of the isolates selected for anti Saprolegnia parasitica activity (table 2) show a broad biological activity spectrum against species of Pythium and Phytophthora as well as antagonist activity against Botrytis, Alternaria and Rhizoctonia.

## Discussion

The results presented here suggest that while selection for antagonists against species of Pythium does yield isolates which are active, these isolates do not show a broad biological activity against other species of Pythium. The use of Saprolegnia parasitica as the target organism does yield isolates which show broad antagonist activity against a variety of Pythium and Phytophthora species. The biological activity spectrum of the antagonists appears to vary with the ecological habitat examined. The relatively broad antagonist activity of some of these isolates against species of Pythium and Phytophthora as well as Botrytis, Alternaria and Rhizoctonia suggests that these isolates should be tested in vivo in soil known to cause damping off. Such isolates might be used singly or in various combinations as a seed dressing for the control of fungi causing damping off.

## Acknowledgements

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A NEW METHOD FOR EVALUATING INTERACTION BETWEEN SOIL INHABITING FUNGI AND PLANT PATHOGENS

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## Introduction

Soil is often strongly colonized by fungi, mainly saprophytic ones. These fungi have, among others, an ecological and phytopathological aspect, the former determined by the structure of their communities, the latter by their effect on plant pathogens, both of value for plant pathological research and practice. In Poland the methodical development in this field led to establishing the so called biotic series method (Mańka, 1974) which aims at determining the effect of the soil environment on a pathogen by estimating the effect of the fungal community inhabiting the soil on the pathogen.

## Methods

The course of work is usually as follows: 1. sampling the soil (when needed also litter, roots, etc.), 2. isolating fungi from it, 3. counting and identifying the isolates obtained, 4. testing the effect of particular isolates on the investigated pathogen, 5. evaluating the summary biotic effect, i.e. a numerical value (positive, negative or neutral) informing about the intensity and quality of the soil's biotic influence on the pathogen.
Ad 1: 6 small samples of soil from different points of soil surface at depth of 5-15 cm are taken, possibly aseptically, mixed and sieved through a 1 mm mashed sieve to obtain the general soil sample.
Ad 2: 1 g of the general soil sample is introduced into a flask containing 149 g of fine quartzy sand and mixed with it by moderate shaking for 2 minutes. Then portions of 25 $mm^3$ of the mixture are transferred into Petri dishes and poured with cooled agar medium according to Johnson (1957), i.e. Czapek-Dox + aureomycine + bengal rose + soil extract. Incubation at room temperature takes 7-10 days.
Ad 4: Testing the effect of particular fungi from the community on the pathogen is conducted in Petri dishes with PDA medium (usually).
It consists of arranging two-fungi cultures in the central part of the dish, the partner fungi placed in yuxta position 2 cm from each other. The inocula are applied in form of agar-culture discs Ø 5 mm (with mycelium turned downwards) or of droplets of spore suspension. After incubation at room temperature for 7-10 days the soil fungus effect on the pathogen is estimated according to the scale presented in Table 1. In situations opponent to those presented in the table the estimates should be of the same absolute value but with minus signs, e.g. -2 instead of +2, etc.

## Results

For presenting results following data must be taken under consideration: 1. frequency of particular species' isolates in the community,
2. the individual biotic effect of the representative isolates (i.e. isolates representing the same fungal species present in the community), 3. the general biotic effects of particular community's species, and 4. the summary biotic effect of the community. The first two data are essential, the further two are their derivatives. The individual biotic effect is obtained by algebraic adding estimation points attributed to a particular isolate representative, and the general biotic effect (of a species) by multiplying the individual effect by the frequency of isolates belonging to the species. Finaly, summarizing general biotic effects the value of the summary biotic effect of the community is obtained (table 2).
As can be seen from table 2, the value of the summary biotic effect is moderately high and negative, what is in accordance with the fact that pine seedlings grown in the soil investigated were infected by Fusarium oxysporum to a moderate extent, too. The values of the summary biotic effects may be much higher, as it reveals from other works of that type.

Table 1. Estimation scale for two-fungi cultures

| Situation in a two-fungi culture | Estimation (points) |
|---|---|
| Both fungal colonies, i.e. of the pathogen (A) and of the soil fungus (B) meeting along a straight line | 0 |
| Colony B meeting colony A along a slightly curved line surrounding less that 1/3 of it | +1 |
| Colony B meeting colony A along a curved line surrounding at least 1/3 but less than 1/2 of it | +2 |
| Colony B meeting colony A along a curved line surrounding at least 1/2 but less than 2/3 of it | +3 |
| Colony B meeting colony A along a curved line surrounding 2/3 or more of it | +4 |
| Each mm of inhibiting zone between partner colonies in favour of colony B | +1 |
| Colony A at least 1/3 but less than 1/2 smaller than its separately grown control colony | +1 |
| Colony A at least 1/2 but less than 2/3 smaller than its seperately grown control colony | +2 |
| Colony A at least 2/3 smaller than its separately grown control colony | +3 |
| Colony A completely undeveloped | +4 |

Table 2. Effect of soil fungi community on <u>Fusarium oxysporum,</u> a pathogen of pine seedlings (Mańka and Kowalski, 1968)

| Soil fungi species | Frequency | Biotic effect Individual | General |
|---|---|---|---|
| Mortierella vinacea | 53 | -3 | -159 |
| Penicillium waksmani | 27 | -6 | -162 |
| Penicillium velutinum | 20 | -5 | -100 |
| Verticillium terrestre | 15 | -1 | - 15 |
| Absidia spinosa | 12 | +3 | + 36 |
| Penicillium lanosum | 6 | +5 | + 30 |
| Trichoderma album | 6 | +5 | + 30 |
| Trichoderma koningii | 6 | +6 | + 36 |
| Pseudogymnoascus vinaceus | 5 | -5 | - 25 |
| Penicillium chermesinum | 4 | -6 | - 24 |
| Penicillium frequentans | 4 | -4 | - 16 |
| Zygorhynchus moelleri | 4 | +6 | + 24 |
| Humicola brevis | 3 | -5 | - 15 |
| Monilia geophila | 3 | -5 | - 15 |
| Mortierella isabellina | 2 | -2 | - 4 |
| Summary biotic effect | | | -445 |

## Final remarks

The method described is being developed and it may be connected in some way with the appeal of Fawcett (1931) for the need of investigation on the effect of saprophytic fungi on fungal plant pathogens. The developments in that area seem also to be bound to Zaleski's (1927/28) achievements in soil mycology, with Worcup's (1950) soil plate method and its modifications (Johnson and Mańka, 1961; Mańka 1974) and some other works.

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# ANTAGONISTIC PROPERTIES OF TRICHODERMA SPECIES AGAINST MYCOCENTROSPORA ACERINA

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## Summary

Fourty *Trichoderma* isolates were screened for antagonism against *Mycocentrospora acerina in vitro*, and their growth at different temperatures and on cellulose was measured. The mechanisms of antagonism were characteristic of different species. In dual culture the viability of *M. acerina* was effectively decreased by *T. viride* and *T. harzianum* type A, which also produced strong non-volatile inhibitors. *T. hamatum* and *T. harzianum* type B were quite ineffective in the production of antibiotics, but some of them aggressively parasitised the hyphae of *M. acerina*. At low temperatures the linear growth rates of the isolates of *T. viride* were superior. The isolates of *T. harzianum* type B were the best utilizers of cellulose as the sole carbon source.

## Introduction

Liquorice rot of carrots caused by *Mycocentrospora acerina* is the most severe storage disease in Finland. The pathogen is soil-borne and colonizes the roots early in the growing season (Wall and Lewis, 1980). The infection arises from chlamydospores on root surface after a few months' storage period (Davies et al., 1981).
Lutchmeah and Cooke (1985) reported a successful control of damping-off caused by *M. acerina* by pelleting seeds with *Pythium oligandrum* in the greenhouse. Pre-storage treatment with *T. harzianum* against storage diseases had a weak effect on *M. acerina* (Tronsmo, 1989). To prevent the colonization of carrot roots by the pathogen, biological control agents should be added in connection with sowing. An essential property for antagonists applied into the soil is the ability to be established in the rhizosphere of carrots. The rhizosphere competence of *Trichoderma* spp. is closely correlated with their ability to use cellulose (Ahmad and Baker, 1988).
The aim of this study was to screen *Trichoderma* isolates of different species *in vitro* against *M. acerina* and to find out some possible mechanisms involved in antagonism. Their ability to grow at different temperatures and on cellulose was measured to indicate their possible growth in control conditions.

## Materials and methods

*Trichoderma* isolates originated mainly from soil and stored carrots, but also from wood. The isolates were identified according to Rifai (1969) and belonged to the species *T. hamatum* (10 isolates), *T. viride* (8) or *T. harzianum*, which was divided into types A (7) and B (15).
Dual culture tests were carried out at 15°C on PDA; the viability of *M. acerina* was tested by transferring mycelial discs from the contact zone and from the area primarily occupied by *M. acerina* on sucrose agar (selective indicator medium for *M. acerina*).

Production of non-volatile inhibitors against *M. acerina* was tested at 15°C on PDA using the cellophane technique (Dennis and Webster, 1971).
Observations of hyphal interactions with 17 representatives from different species were made on a cellophane membrane over water agar with phase-contrast microscopy.
Growth at different temperatures was measured as radial growth on PDA and the ability to use cellulose as dry weight of mycelium grown in Czapek-Dox broth with 2 % microcrystalline cellulose as the sole carbon source at 20°C.

## Results

In dual culture tests all isolates prevented the growth of the pathogen and overgrew it. The most complete covering of *M. acerina* colonies was achieved with *T. harzianum* A isolates. *T. viride* and *T. harzianum* A significantly decreased the viability of *M. acerina*; especially the inhibition by *T. viride* was complete.
The isolates with strong inhibition ability produced effective non-volatile antibiotics. After one week of incubation *T. harzianum* A and *T. viride* inhibited of 98 and 88 % of the growth of *M. acerina*, respectively, compared with control. Only in few cases the pathogen started to grow during the next month. Non-volatile metabolites of *T. hamatum* and *T. harziamun* B isolates inhibited the pathogen only slightly, 31 and 21 %, respectively, after one week, and soon all plates were filled by the pathogen (Table 1).
When the hyphal interactions were studied, most isolates of *T. hamatum* and *T. harzianum* B grew intensively toward *M. acerina* hyphae and coiled around it. *T. harzianum* A seemed to be indifferent to *M. acerina* at least at the early stages when mycelia grew among the pathogen.
At 20°C the radial growth rates of *T. harzianum* A and B were the most rapid, but at 15°C *T. harzianum* A and *T. viride* were the best growers. All isolates of *T. viride* grew at 2°C, on average 25 mm during a month, while only 3 of 10 isolates of *T. hamatum* and 2 of 7 isolates of *T. harzianum* A were able to grow more than 7 mm during a month. *T. harzianum* B isolates grew hardly at all at 2°C (Table 1).

Table 1. Inhibition of the growth of *M. acerina* with non-volatile antibiotics produced by *Trichoderma* isolates, and the growth of *Trichoderma* isolates at 2°C and on microcrystalline cellulose as sole carbon source.

| Species | Number of isolates | Inhibition with antibiotics % *) | Growth at 2°C, % of the isolates **) | Growth on cellulose mg/week |
|---|---|---|---|---|
| *T. hamatum* | 10 | 31 | 30 | 155 |
| *T. harzianum* A | 7 | 98 | 29 | 169 |
| *T. harzianum* B | 15 | 21 | 0 | 282 |
| *T. viride* | 8 | 88 | 100 | 167 |

*) inhibition of the growth of *M. acerina* after incubation of one week at 15°C
**) radial growth on PDA more than 7 mm during a month

On average the isolates of *T. harzianum* B produced the greatest mycelium yield on cellulose (282 mg). One *T. harzianum* B isolate (M01-4) had significantly higher growth rates on cellulose (479 mg) than the other isolates. The amount of produced biomass of the other species was about the same, on average 164 mg (Table 1).

## Discussion

Obviously, all tested *Trichoderma* isolates are hyperparasites, but in the case of *T. harzianum* A and *T. viride* antibiosis is probably the main mechanism of antagonism, while *T. hamatum* and *T. harzianum* B were mainly aggressive parasites.
Although *T. viride* isolates grow quite well at low temperatures as also reported by Tronsmo and Dennis (1978), they are much slower than *M. acerina*, which can grow even at -3°C (Gündel, 1976).
The relative differences in the growth rates between *Trichoderma* isolates on cellulose probably also correlate with their rhizosphere competence, compared with the results of Ahmad and Baker (1988) on benomyl resistant mutants and wild *Trichoderma* isolates. According to Sivan and Chet (1989) a wild *Trichoderma* sp. also has rhizosphere competence.
The work will continue to find out which of these properties correlate with antagonistic abilities *in vivo*.

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# Screening of fungal antagonists to control Sclerotium cepivorum

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## Summary

Ten fungal species were selected from a total of 26 on the basis of their antagonism to Sclerotium cepivorum in dual culture in vitro. The 10 fungal species selected identified as Trichoderma spp., competed successfully with the pathogen for space and nutrients.

## Introduction

Allium white rot, caused by the soilborne fungus S. cepivorum Berk. is a widespread disease reported from most countries where Allium spp. are cultivated. In Spain the disease severely affects onion (Allium cepa), garlic (Allium sativum) and leek (Allium porrum) resulting in significant yield losses (Vares et al., 1987).

Sclerotia, the only propagule of S. cepivorum, are able to persist in soil for long periods of time (Coley-Smith, 1959) and may induce high disease incidences at low inoculum densities (Crowe et al., 1980). In Southern Spain the use of fungicide treatments (calomel, benomyl, iprodione) have proved ineffective in eradicating the disease. Microbial control is an alternative method which consists of the application of antagonists, either to the soil or to the plant, to control Allium white rot. A number of soil microorganisms including Trichoderma harzianum, Penicillium nigricans, Coniothyrium minitans, Gliocladium roseum, Bacillus subtilis have been identified as antagonists of S. cepivorum (Ahmed & Tribe, 1977; Abd-El-Moity & Shatla, (1981); Oliveira et al., 1984; Harrison & Stwart, 1988); Backhouse & Stewart, 1989).

The objective of the present study was to isolate fungal antagonists of S. cepivorum and assess, in vitro, their ability as biological control agents against this pathogen.

## Materials and Methods

### Growth and maintenance of isolates

#### Sclerotium cepivorum

Active cultures of S. cepivorum. Naturally produced sclerotia were surface-sterilized for 3 min in sodium hypochlorite (20% v/water) and later rinsed twice in sterile distilled water for 5 min. The sclerotia were then placed on Oxoid malt extract agar plus antibacterium. After incubation for four days at 18ºC, mycelium from germinating sclerotia was transferred to Petri dishes containing Difco potato-dextrose agar. These plates were incubated for six days before being used for experimental purposes.

#### Antagonistic fungi

Stock cultures of antagonistic fungi were maintained at 18ºC in glass tubes containing PDA. Active cultures were obtained by transfer of mycelium to plastic Petri dishes with PDA. The antagonistic fungi were incubated for six days at

18ºC before being used for experiments.

Dual culture

Antagonism on agar plates was studied using the methods described by Royse & Ries (1878) and Whipps (1987). Twelve possible antagonists were studied. A 5 mm mycelial plug of the potential antagonist was placed diametrically opposite a 5 mm plug from an active culture of S. cepivorum. The distance between plugs was 5 cm. Five replicates were used for each combination and the plates were incubated for 18 days at 18ºC. Inhibition of the pathogen's growth was determined by measuring the percentage inhibition of radial growth (%IRG) after 4, 11 and 18 days as:

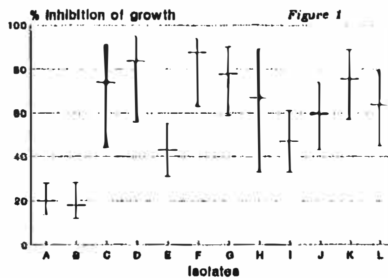
$$\%IRG=100x[(R1-R2)/R1]$$

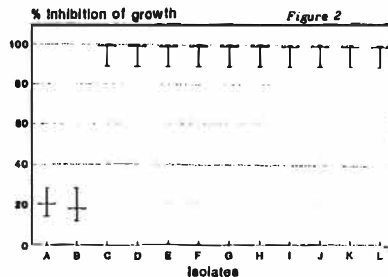
R1 is the furthest radial distance grown by the pathogen in the direction of the antagonist, while R2 represents the distance grown on a line between inoculation positions of the pathogen and the antagonist. The data were analyzed by analysis of variance.

## Results and Discussion

Dual culture.

Fig. 1 reveals the percentage of inhibition of growth by 12 fungal isolates on PDA after 10 days. Isolates A and B correpond to two of the sixteen isolates which were not selected as possible antagonists of S. cepivorum. The rest of the isolates, identified as Trichoderma spp. showed a good level of inhibition of growth after 10 days. After 17 days all Trichoderma spp. showed a level of inhibition of growth of 100%, whereas isolates A and B did not inhibit further the growth of S. cepivorum (Fig.2).




*Figs. 1-2. Percent of inhibition of radial growth of S. cepivorum after 10 (fig.1) and 17 days (fig.2) at 18º C by different fungal isolates.*

These results should be interpreted with care since it has been shown that different nutritional media may affect the competition between pathogen and antagonists (Whipps, 1987). Further research needs to be carried out using different media and environmetal variables to know the potential of these organisms to be effective antagonists against *S. cepivorum.*

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# PROSPECTS FOR BIOCONTROL OF ARMILLARIA MELLEA USING MUSHROOM PATHOGENS.

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## Summary

Pathogens of Agaricus bisporus are currently undergoing investigation as possible biocontrol agents of another hymenomycete Armillaria root rot of trees. Initial findings showed **in vitro** antagonism of honey fungus by Trichoderma harzianum and Dactylium dendroides on 3% malt extract agar. This significant difference was maintained over a range of pH and temperatures. Work on sawdust agar has supported this. Further work is being carried out to investigate the **in vivo** situation.
Current study is evaluating the production of inhibitory metabolites by Trichoderma harzianum and their role in the control of A. mellea. Especially, whether different strains show different characteristics in the level of inhibition of Armillaria spp. growth.

## Introduction

The genus Armillaria causes important root rots of trees resulting in the destruction of trees in orchards, plantations and woodland. Previous chemical methods to control it have relied on stump drenching or soil fumigation. Chemical treatments have included carbon disulphide, 2,4,5 trichlorophenoxyacetic acid (Garrett 1958) and recently ergosterol biosynthesis inhibitors (Turner and Fox 1988).
In the present climate of public opinion chemicals have lost their appeal as a method of control. Armillaria spp. represents a good candidate for biocontrol because it has a limited ability to compete with other organisms. Previously biocontrol have included Trichoderma viride (Garrett 1958) and cord formers such as Hypholoma spp. (Dowson, et **al** 1988).
This work covers the use of mushroom pathogens, to control Armillaria spp., such as Trichoderma harzianum and Dactylium dendroides (Fox et al 1991).

## Materials and Methods

The work was carried out on A. mellea. The mushroom pathogens were Trichoderma harzianum, Dactylium dendroides, Chaetomium olivaceum, Aphanocladium album, Myceliophora lutea, Deranomyces stemotis and some specimens isolated from the field referred to as PA1, PA2, PA3, PA4. Investigations were carried out on 3% malt extract agar in which temperature, pH, and variation of media to potato dextrose media were all evaluated. Following challenge with potential antagonists A.mellea colonies were tested for viability by subculturing on to fresh malt extract agar. As a move towards field conditions experiments were repeated on 10% beech sawdust. The viability experiments have led to the idea of a toxic metabolite being produced this was investigated using

nitrocellulose overlay filters on 3% malt extract agar and organic solvent extractions from liquid cultures of the antagonists.

## Results

### Malt extract

The most effective antagonists were Trichoderma harzianum and PA1. The effect of all the biocontrol agents was highly significant. See Figure 1. Early results indicated that the mushroom pathogens Aphanocladium album, Deratomyces stemotis and Myceliophora lutea had no effect on the growth of A. mellea (Fox et al. 1991).
Testing a wide range of conditions revealed that PA1 was most effective at the optimum conditions for A. mellea that is at low pH (pH 5) and at temperatures in the range of 15-20°C. Trichoderma harzianum had a wide range of activity over pH 5-8 and 15-25°C. Changing the media did not change the pattern of antagonism although the colony size of A. mellea was smaller due to differences in pH. Only one of the mushroom pathogens, Chaetomium olivaceum tested did not appear to have a cidal effect on Armillaria spp.. This led on to the theory that the antagonists may produce some form of a toxic metabolite.

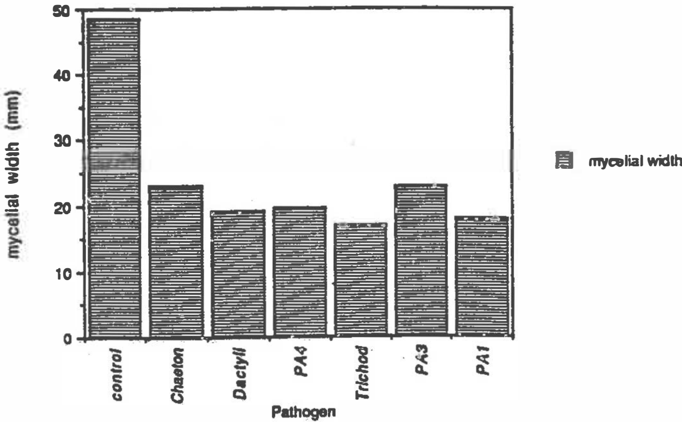


Figure 1. Quantitative analysis of inhibition by mushroom pathogens on the growth of A. mellea

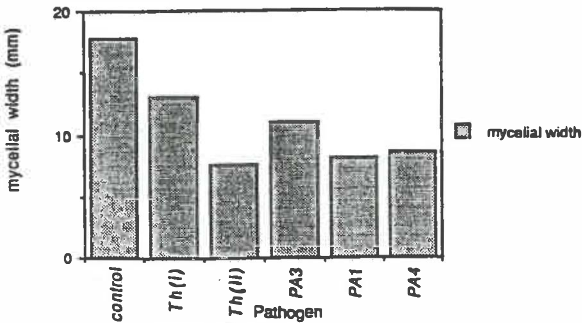


Figure 2. The effect of antagonism by mushroom pathogens on A. mellea on sawdust agar.

## Sawdust experiments

The following antagonists grew on sawdust agar Chaetomium olivaceum , Trichoderma harzianum (strains 1 & 2) , PA1, PA3 and PA4. All showed antagonism to Armillaria mellea on sawdust. The most effective were PA1 and Trichoderma harzianum 2. (Figure 2.) The effect of the antagonists was at a significant level and followed the same pattern as had been observed on 3% malt extract agar. The reduction in A. mellea was in the range of 50%.

## Discussion

Two highly effective biocontrol agents have been found, Trichoderma harzianum and isolate PA1 which is a "Dactylium dendroides type" organism. The majority of the work was carried out on malt extract agar, however the organisms exert control over a wide range of pH and temperature. This is necessary as Armillaria spp. flourish in a wide range of conditions. The viability experiments implied that there was some kind of cidal effect from the mushroom pathogens, this will be addressed more closely with the extraction of toxic metabolites.
The sawdust experiments implied that Trichoderma harzianum has shown good potential as a biocontrol because it has a rapid colonisation of substrate. PA2 is active at lower temperatures such as those found in the field. Both the Trichoderma harzianum and PA1 were effective antagonists on sawdust.

## Acknowledgements

This work was supported by sponsorship from the Ministry of Agriculture, Food and Fisheries, U.K.

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## EFFECTS OF *TRICHODERMA* AND *PENICILLIUM* ISOLATES ON TULIP TIP-BURN LEAF DISORDER

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**Abstract**

The effects of one *Penicillium* and two *Trichoderma* isolates on tulip tip-burn leaf disorder were tested by applying the isolates to unsterilized and sterilized tulip growing media. The *Penicillium* isolate and one of the *Trichoderma* isolates were able to increase tip-burn incidence under certain conditions, while the other *Trichoderma* isolate as well as autoclaved barley totally inhibited tip-burn outbreaks and thus worked as control agents.

**Introduction**

Symptoms - named tip-burn - showing grey, wilting and shrivelling leaf tips, at flowering time in forced tulips, *Tulipa gesneriana* L, have long been a problem in certain Swedish growings. The symptoms occur more frequently in some years and are especially common in some cultivars. In Denmark this kind of leaf disorder was found to be caused by excess of boron (Rasmussen, 1974), but in Sweden it is assumed to be the result of *Trichoderma viride* Pers. ex. Grey infections in the roots, as was stated by Muller et al. (1979) and by Conijn and Muller (1981).

The experiments reported here give evidence that tip-burn incidence is affected by amending the growing medium with laboratory-grown *T. viride* and *Penicillium* Link ex Fr. sp. isolates, but also that it may be either increased or decreased by such amendings, depending on isolate and/or conditions applied.

**Materials and methods**

Tulips (cultivar "Prominence") were grown in a suitably fertilized, commercially sold *Sphagnum* peat (Hasselfors Lökmull) in plastic boxes (60 x 40 x 8 cm) with about 100 bulbs per box. The planted boxes were placed in dark chambers at 3 - 6° C and 90 - 100 % RH for about ten weeks and were then moved to a greenhouse where the percentage of plants with symptoms were read when the tulips were at full flowering (after about one month). The sterilization of the growing medium (as used in some treatments) was done by autoclaving at 121° C for 30 minutes on two consecutive days. Surface sterilization of bulbs (as used in one treatment) was done by carefully washing them in water then dipping them in 70 per cent ethanol and finally washing them several times in sterile water.

The fungal isolates tested, one *Penicillium* sp. (P 2) originating from a tulip bulb and two *T. viride*, one from a tulip root (T 30) and the other from a field soil (T 18) were grown on autoclaved barley in plastic bags at about 25° C and under continuous light. They were grown for about three weeks until sporulating profusely and were

then dried and stored until used for thoroughly mixing with the tulip peat growing medium (about 1:20 v/v) at the time of planting.
Three experiments with two boxes per treatment were carried out 1983-1985. The boxes were used as repetitions and results from two or three experiments were combined in those cases where the different experiments contained the same treatment.

## Results

Percentage of tulip plants showing symptoms in the various treatments applied are shown in Figure 1.

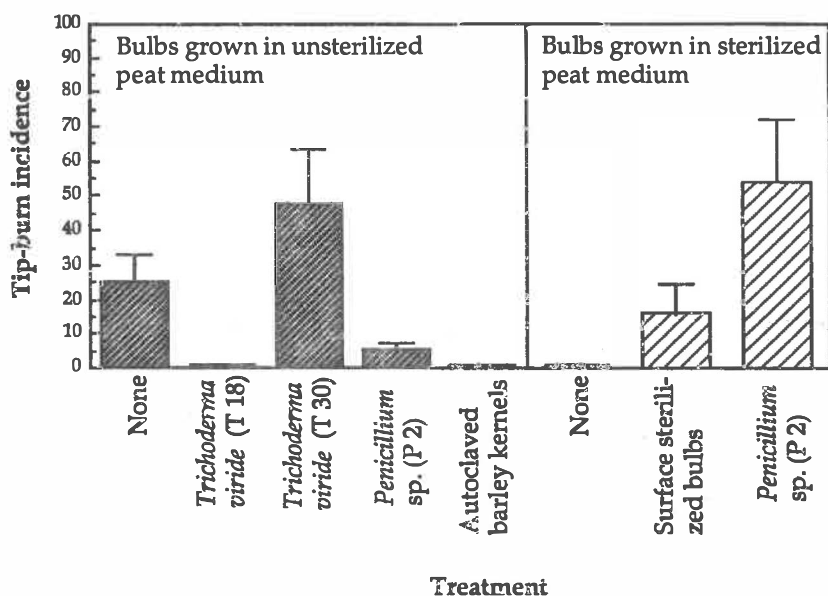


Fig. 1. Percentage of plants with tip-burn symptoms at full flowering in the various treatments applied. Small bars represent standard error.

## Discussion

Our findings of i) a clear difference between the two tested *T. viride* isolates in their effect on symptom frequency, ii) different effects of *Penicillium* in unsterile and sterile growing media, and iii) the total suppression of symptoms by autoclaved barley (Fig. 1), all point to a microbial background to the symptoms read. If *T. viride* is causing the tip-burn, as was stated by Conijn and Muller (1981), its effect must be very sensitive to the microbial balance in or around bulbs and/or roots. However, our findings of several factors affecting outbreaks indicate that *T. viride* infecting the roots probably is not the main or the only cause. The high incidence of tip-burn in the sterilized and *Penicillium*-amended growing medium points to the tested *Penicillium* isolate as a causing agent. However, the low incidence obtained when

*Penicillium* was supplied to unsterile growing medium, again indicates an involvement of microbial interactions and an indirect effect. Therefore, from our results we find it hard to sort out any single microbial agent. Our results do not either allow a sorting out the origin of a possible causing microbial agent. The total disappearance of symptoms when the tulips were grown in sterilized medium supplies evidence that the causing agent occurs in the growing medium. However, the high symptom incidence obtained when we also surface sterilized the bulbs disagrees to such an explanation. Two other possibilities are then likely: i) that tip-burn still has its origin in the growing medium, but we had a reinfection of the surface sterilized bulbs, and that unsterilized bulbs had a natural flora suppressing such reinfections or ii) that a tip-burn inducing agent has the origin in the bulbs and that there are antagonists on them, which in sterilized medium and if not killed by surface sterilization suppress this agent. The agent itself would in this case not be sensitive to sterilization, i. e. be situated deeply inside the bulb.
Such an occurrence of a causing agent and also natural antagonist(s) on or in the bulbs would agree well with the finding that autoclaved barley totally suppressed the symptoms. The autoclaved barley would then stimulate the bulb antagonists. Such an effect could also explain the good effects obtained with the *Trichoderma* isolate T 18, on the assumption that this isolate was neutral to both the antagonist(s) and the symptom-causing agent, but the barley on which it was grown stimulated antagonists. However, T 18 may also in itself be a very effective antagonist. The symptom-increasing mechanism exerted by the isolates T 30 and P 2 seems in any case to be either a direct pathogenicity, an interference with another symptom-causing agent increasing its activity or, as a third possibility, a suppression of its antagonist(s).
The results obtained indicate certain possibilities of using biological control or soil amendments as control measures in commercial growings. However, the tests hitherto done are far too inadequate to be used as a background for specifying any recommendations to growers.

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# A TOXICOLOGICAL APPROACH TO THE BIOCONTROL OF PLANT PATHOGENS BY TRICHODERMA.

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## Summary

The toxicity of 9 antagonistic Trichoderma isolates was examined. Culture extracts were tested in brine shrimp (Artemia salina L.) larvae mortality assays to evaluate mycotoxic activity (toxicity toward animals), in tomato seedling assays to test phytotoxicity, and in Geotrichum candidum Link growth inhibition assays to test antibiotic activity. Some of the tested extracts were observed to be highly toxic toward A. salina, causing 100% larvae mortality at an extract dose equivalent to 4.5 ml culture filtrate/ml sea water. The phytotoxicity proved to be appreciable, achieving for some isolates up to 55% reduction in tomato seedlings rootlet lenght at an extract dose equivalent to 1.5 ml culture filtrate/ml water. Antifungal metabolites, active against G. candidum were produced by all the tested isolates. These preliminary results showed a difference in the toxicity among isolates with the same antagonistic activity. The toxic isolates potentially useful for the biocontrol of plant pathogens should be tested with ad hoc biological monitoring systems depending on their specific employment, and possible toxicological risks should be considered.

## Introduction

It is well estabilished that certain antagonistic fungi are able to produce toxic metabolites which can assume the significance of chemical weapons in microbial competition. Biocontrol application of toxic fungi or their metabolites having a wide spectrum of toxic activity may interfere negatively on the biological environment considered.

Trichoderma is a well-known antagonist of both soil-borne and air-borne plant pathogens. Antifungal compositions containing Trichoderma have been patented and their practical application is under experimental observation in some countries.The mechanism by which Trichoderma exerts its antagonistic activity is not yet clear, but it is proved that Trichoderma produces and secretes toxic metabolites (antibiotics, toxins) during its growth and possibly also during its antagonistic action (Brewer et al., 1982). Therefore, the introduction and diffusion of natural or improved Trichoderma strains in the environment could represent a risk that should be evaluated.

A toxicological approach to the biocontrol of plant pathogens by Trichoderma needs to test the antagonistic strains by both general aspecific bioassays and ad hoc biological monitoring systems in order to assess their toxicological pattern. In this preliminary study, 3 toxicity bioassays were used: Brine shrimp (Artemia salina L.) larvae mortality assay, a simple sensitive method widely employed to estimate the toxic potential of mycotoxins, pesticides and

other poisonous compounds (Harwig & Scott, 1971), in order to test Trichoderma mycotoxic activity (toxicity toward animals); tomato seedling root inhibition assay to test phytotoxicity; Geotrichum candidum Link ex Pers. growth inhibition assay to test antifungal activity.

## Materials and Methods

All the bioassays were performed using the ethyl acetate soluble fractions of Trichoderma cultural broths.

1. Brine shrimp assay.

Bioassays were performed in cell culture plates (Corning, N.Y.) with 24 wells containing about 30-40 brine shrimp (A. salina) larvae in 500 µl sea water with 1% culture extract per well (4 replicates per extract). The number of dead shrimps was recorded after incubation at 27 °C for 36 hr. The total number of shrimps per well was measured after killing the remaining shrimps by freezing at -20 °C for 12 hr and the per cent larvae mortality was calculated.

2. Tomato seedlings assay.

Tomato seeds were surface sterilized with 4% NaClO for 10 min. and left to germinate on water impregnated filter paper for 3 days at room temperature in the dark. The germinating seedlings, selected for sanity and evenness were put into a Petri dish (5 cm) containing a same size filter paper. Two ml of water containing 1% ethyl acetate (control) or Trichoderma culture extracts were added, and root elongation measurement were made after 4 days of incubation at room temperature. Three replicates per test were performed.

3. Geotrichum candidum assay.

Trichoderma culture extracts were adsorbed on 6 mm concentration disks (Difco, Detroit, USA). After solvent evaporation, the disks were laid on potato-dextrose-agar (PDA) plates and sprayed with a mycelium suspension of G. candidum. The antifungal activity was evaluated after 24 hr by the fungal growth inhibition halo.

## Results and Discussion

The data of brine shrimps larvae mortality exposed to Trichoderma culture extracts showed differences among the isolates with regard to their mycotoxic activity. Such differences ranged from not statistically different to the control ($P = 0.01$), to 100% mortality for some extracts. The phytotoxicity proved to be appreciable, achieving for some isolates an up to 55% reduction in tomato seedlings root lenght ($P = 0.01$). The antifungal activity against G. candidum was shown to be equally high for all the tested isolates. The positive correlation between the antagonistic activity of the isolates and the antifungal activity of their extracts strongly suggested the involvement of metabolic products in the mechanism of biocontrol.

These preliminary results showed a difference in the toxicity among isolates with the same antagonistic activity (Table 1). The toxic isolates potentially useful for the biocontrol of plant pathogens should be tested with ad hoc biological monitoring systems (bioindicators) depending on their specific employment, and possible toxicological risks should be considered.

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TABLE 1: Toxicity of Trichoderma strains in different bioassays (a).

| Strain (b) | A. salina (c) | | tomato rootlet lenght (d) | | G. candidum (e) | |
|---|---|---|---|---|---|---|
| Control | 10.9 | A | 37.6 | AD | 0 | |
| ITEM 917 | 11.4 | A | 36.9 | AD | 14.4 | bc A |
| ITEM 918 | 100.0 | D | 47.3 | A | 15.2 | bc A |
| ITEM 919 | 100.0 | D | 19.8 | E | 16.4 | c A |
| ITEM 920 | 100.0 | D | 17.0 | E | 15.7 | c A |
| ITEM 921 | 35.4 | B | 33.6 | BD | 9.5 | a A |
| ITEM 922 | 13.2 | A | 43.4 | AC | 15.6 | bc A |
| ITEM 923 | 100.0 | B | 28.6 | CE | 17.0 | c A |
| ITEM 924 | 13.9 | A | 39.2 | AD | 15.6 | bc A |
| ITEM 925 | 90.8 | C | 28.8 | CE | 11.9 | ab A |

(a) - Means in columns followed by same letter are not significantly different according to Duncan's multiple range test at P = 0.01 (capital letters) and P = 0.05 (small letters).

(b) - Trichoderma strains conserved in the Istituto Tossine e Micotossine (ITEM) collection.

(c) - Per cent larvae mortality at an extract dose equivalent to 4.5 ml culture filtrate / ml sea water.

(d) - Rootlet lenght (mm) at an extract dose equivalent to 1.5 ml culture filtrate / ml water.

(e) - Inhibition halo diameter (mm) at an extract dose equivalent to 3.0 ml culture filtrate / disk.

# BACTERIAL ANTAGONISTS

## BACTERIAL ANTAGONISTS OF PLANT PATHOGENS IN THE RHIZOSPHERE: MECHANISMS AND PROSPECTS

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Among a range of rhizobacteria, including *Agrobacterium*, *Bacillus* and other genera, with the ability to promote plant growth through biocontrol or other mechanisms, the pseudomonads in particular have received worldwide attention (Burr and Caesar, 1984; Défago and Haas, 1990; Schippers, 1988; Weller, 1988). Inasmuch as the pseudomonads appear to be the most important group of antagonistic bacteria in soil, this review will focus on some aspects in the large field of studies on these bacteria and on their beneficial effects.

### DISEASE SUPPRESSION BY PSEUDOMONADS

Bacterial biocontrol agents improve plant growth by suppressing either major or minor pathogens (Défago and Haas, 1990; Weller, 1988). Major pathogens cause well-known diseases with characteristic symptoms. In general, a *Pseudomonas* strain is able to suppress more than one disease, e.g. *P. fluorescens* CHA0, which was isolated from a naturally suppressive Swiss soil (Stutz *et al.*, 1986). Strain CHA0 was found to be an effective biocontrol agent of various soilborne diseases caused by *Thielaviopsis basicola*, *Fusarium oxysporum* spp., *Rhizoctonia solani*, *Pythium ultimum* and other pathogens in artificial and natural soils (Défago *et al.*, 1990). The same strain suppresses take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* in greenhouse and field experiments. Wheat health was improved in most, but not all, field trials conducted over several years (Défago *et al.*, 1990; Wüthrich and Défago, 1991).

Minor pathogens include saprophytes or parasites that cause damage to the plant, but without disease symptoms being obvious (Alström, 1991). Bakkers and Schippers (1987) hypothesized that reduction of potato yields in Dutch fields is due to the presence of rhizobacteria producing deleterious substances (e.g. HCN). Potatoes grown in the same field every third year (short rotation) yielded about 15% less than when grown every sixth year (long rotation). Application of pseudomonads to field trials promoted potato growth in the short rotation cropping soil, presumably by suppressing the deleterious bacteria (Schippers *et al.*, 1987).

In some cases pseudomonads promote plant growth in the absence of pathogenic microorganisms. The enhanced plant growth response may be caused by an increased availability of certain mineral nutrients or by the production of plant growth regulators. Lifshitz *et al.* (1987) suggested that increased root elongation of rape seedlings was due to improved phosphate uptake induced by a *P. putida* strain. However, direct growth promotion was found only under axenic or gnotobiotic conditions, but never in the field; thus, it is generally accepted that the beneficial effect of the pseudomonads arises mainly from the suppression of major or minor pathogens.

### ROOT COLONIZATION

**Properties of a rhizosphere competent bacterial antagonist.** An effective biocontrol agent needs to colonize the whole root system intensively, in the sense of being present at the sites where the infection takes place. This means that after introduction it becomes distributed along the root, propagates, survives and exhibits its beneficial effect for several weeks in the presence of competition from the indigenous rhizosphere microflora (Parke, 1990; Weller, 1988). Among the complete range of *Pseudomonas* strains found in the rhizosphere, only a few possess these attributes. Some *Pseudomonas* strains, e.g. CHA0, were found not only on the root surface but also in the root cortex, as shown by indirect immunofluorescence staining; small lesions in the root surface were used by strain CHA0 for intrusion into the cortex (Défago *et al.*, 1990).

**Factors influencing root colonization.** Root colonization is influenced by characteristics (rhizosphere competence traits) of the introduced pseudomonad, the rhizosphere microflora, the plant, and the abiotic environment. Bacterial phenazine antibiotic production was shown to contribute to the long-term survival of fluorescent pseudomonads in soil (Thomashow and Pierson III, 1991). The production of specific siderophores and the utilization of a broad spectrum of siderophores contributes to the root-colonizing and plant growth-promoting ability of a *P. putida*

strain (Bakker *et al.*, 1986). Chemotactic attraction of pseudomonads towards root- or seed-exudates (Bashan, 1986; Scher *et al.*, 1985) may be a guide to infection sites where exudation is enhanced. Tolerance to dry soil and low osmotic potential may help some introduced bacteria to survive (Howie *et al.*, 1987; Loper *et al.*, 1985). Studies with flagella-negative, nonmotile Tn*5* mutants revealed that motility either enhanced (De Weger *et al.*, 1987) or had no effect (Howie *et al.*, 1987) on root colonization. Cell surface properties, like agglutinability (Anderson *et al.*, 1988; Glandorf *et al.*, 1991), lipopolysaccharides (De Weger *et al.*, 1989), and pili (Vesper, 1987) may help to support the establishment or the specificity of bacteria-plant associations. Composition of root exudates may vary with species, cultivar, and even growth stage of the plant and thus affect root colonization (Weller, 1988). Furthermore, interactions with the indigenous rhizosphere microflora and physical and chemical characteristics of the soil may drastically influence the establishment and survival of an introduced biocontrol agent (Davies and Whitbread, 1989; Weller, 1988).

## MECHANISMS OF PATHOGEN SUPPRESSION

The capacity to colonize the roots intensively for an extended period of time is not sufficient by itself for a pseudomonad to be an effective biocontrol agent. Other mechanisms are involved. Inhibition of the pathogen by releasing diffusible or volatile metabolites (antibiosis) or by competition for micro- or macronutrients (e.g. siderophore-mediated iron competition), degradation of pathogen toxins and induction of plant defence mechanisms against the pathogen are currently proposed as major mechanisms involved in disease suppression (Défago and Haas, 1990; Fravel, 1988; Weller, 1988). The diversity of possible mechanisms is based on the wide array of secondary metabolites produced by pseudomonads which may be important in disease suppression (Défago and Haas, 1990; Kiprianova and Smirnov, 1981; Leisinger and Margraff, 1979).

**Siderophore-mediated competition for iron.** When grown under iron-limited conditions fluorescent pseudomonads produce fluorescent, yellow-green siderophores (pyoverdine, pseudobactin) that function as high-affinity $Fe^{3+}$ chelators. According to the siderophore hypothesis, pseudomonads suppress disease by sequestering the limited supply of iron in the rhizosphere and thereby limiting the availability of the iron necessary for the growth of the pathogens. Support for this hypothesis has come from studies on siderophore-minus mutants and on the effects of purified siderophores or synthetic iron chelators which presented evidence of an involvement of pyoverdines in the biocontrol of diseases caused by *F. oxysporum* spp., *P. ultimum* and minor pathogens (Loper and Buyer, 1991). However, an increasing number of reports indicate that bacterial metabolites other than siderophores, *i.e.* antibiotics, do have a key role in disease suppression (Haas *et al.*, 1991; Keel *et al.*, 1989; Kraus and Loper, 1991; Thomashow and Weller 1990). A pyoverdine-negative Tn*1733*-mutant of *P. fluorescens* CHA0 protected tobacco and wheat from rot root diseases with wild-type efficiency both in natural and artificial soil, whether plants were grown under conditions of iron sufficiency or deficiency (Défago *et al.*, 1990; Haas *et al.*, 1991; Keel and Défago, 1991; Keel *et al.*, 1989).

**Antibiosis.** Recent progress towards understanding this mechanism has resulted mainly from the application of DNA technology and biochemical research techniques (Défago and Haas, 1990; Fravel, 1988). Chemically or genetically generated mutants that were defective in the production of specific compounds, e.g. oomycin A, pyrrolnitrin, pyoluteorin, phenazines or cyanide, were tested for disease suppression (Gutterson, 1990; Homma and Suzui, 1989; Kraus and Loper, 1991; Thomashow and Weller, 1988; Voisard *et al.*, 1989). Tn*5* insertion mutants of *P. fluorescens* 2-79 defective in phenazine-1-carboxylic acid (PCA) production had a reduced capacity to suppress take-all of wheat (Thomashow and Weller, 1988). PCA production and disease suppression were restored in the mutants by complementation with cloned PCA biosynthetic genes. Furthermore, PCA could be detected in the rhizosphere of wheat, *i.e.* at the site of take-all suppression (Thomashow *et al.*, 1990). HCN-negative mutants of *P. fluorescens* CHA0, obtained by gene replacement techniques, suppressed tobacco black root rot with reduced efficiency under gnotobiotic conditions. A recombinant plasmid complemented for HCN production and restored disease suppression (Haas *et al.*, 1991; Voisard *et al.*, 1989). A Tn*5*-mutant of CHA0, defective in synthesis of the antibiotic and phytotoxic compound 2,4-diacetylphloroglucinol (Phl) was significantly less effective in suppressing black root rot of tobacco and take-all of wheat. The mutant was not able to produce Phl in the rhizosphere of wheat. Complementation of the mutant

by a recombinant cosmid containing a DNA fragment from wild-type CHA0 largely restored its capacity to produce Phl *in vitro* and in the rhizosphere of wheat and to suppress disease (Keel *et al.*, 1990, 1991).

**Degradation of pathogen toxins.** Toyada *et al.* (1988) found a nonpathogenic mutant of *P. solanacearum* which was able to detoxify fusaric acid, the wilt toxin of *Fusarium oxysporum* f. sp. *lycopersici* and to protect tomato plants from the wilting disease. Extracellular proteases of fluorescent *Pseudomonas* strains are suggested as inactivating hydrolases and phytotoxins of phytopathogenic *Fusarium* spp. (Borowicz *et al.*, this volume; Pietr, 1991).

**Induction of plant defence mechanisms.** There is an increasing number of reports indicating that pseudomonads might also suppress diseases by inducing resistance in the plant. Bacterial metabolites, like HCN or 2,4-diacetylphloroglucinol, may also be toxic to the plant (Keel *et al.*, 1990; Voisard *et al.*, 1989). Subinhibitory quantities of the metabolites produced by the bacteria closely associated with the root might induce the stress necessary to activate plant defence mechanisms against the pathogen (Défago *et al.*, 1990; Keel *et al.*, 1990).

In our experiments we found a bacterial influence on the plant also in the absence of the fungal pathogen (Défago *et al.*, 1990). In the presence of *P. fluorescens* CHA0, tobacco roots were stunted and showed an increased root hair formation. Additional experiments showed that the morphological changes of tobacco roots were correlated with the presence of functional cyanide genes in the bacteria (Défago *et al.*, 1990). These genes, as shown above, are necessary for an efficient disease suppression (Voisard *et al.*, 1989). Thus, it seems possible that bacterial HCN might modify plant metabolism in a way that induces some defence mechanisms. Van Peer and Schippers (1991) have investigated the possible involvement of induced resistance in biological control of *F. oxysporum* f. sp. *dianthi* on carnation by fluorescent *Pseudomonas* sp. WCS417. Roots were bacterized one week prior to stem-inoculation with conidia of *Fusarium*. Disease was significantly reduced when plants were bacterized compared to plants inoculated with *Fusarium* alone. Wei *et al.* (1991) have adapted the classical cucumber-*Colletotrichum lagenarium*-system used by Kuc *et al.* (1975) for investigations into induced systemic resistance. They treated cucumber seeds with beneficial bacterial strains and later challenged with *Colletotrichum* by inoculating the second leaf with a conidial suspension (Wei *et al.*, 1991). Some of the bacterial strains reduced lesion number and lesion size as compared to the noninduced control.

Although these systems provide evidence for an involvement of induced resistance, they cannot rule out the possibility that bacterial metabolites, e.g. antibiotics, could be taken up and translocated in the plant and directly antagonize the pathogen, exerting a systemic protection comparable to the action of certain pesticides.

## INFLUENCE OF ENVIRONMENTAL FACTORS

Both root colonization (see above) and production and activity of bacterial metabolites involved in pathogen suppression may be affected by the physical and chemical characteristics of the soil (e.g. clay minerals, pH, iron).

The Swiss soil which is naturally suppressive to black root rot of tobacco caused by *T. basicola* is part of a thick, weathered ground moraine of the Rhone glacier and contains mostly vermiculitic clay minerals; the neighbouring conducive soil consists of weathered molasse containing mostly illitic-smectitic clay minerals (Stutz *et al.*, 1985, 1989). Iron is necessary for HCN production by pseudomonads, e.g. by *P. fluorescens* CHAO (Voisard *et al.*, 1989). Addition of iron to batch cultures of strain CHA0 in minimal medium induced HCN production. When iron was replaced by pure vermiculite, cyanogenesis was also induced. In contrast, when illite was added instead of vermiculite or iron, HCN production remained poor (Keel *et al.*, 1989). In addition, the degree of protection afforded by CHA0 was distinctly greater in vermiculite soils than in illite soils (Keel *et al.*, 1989; Stutz *et al.*, 1989). However, the addition of iron to the illite soil allowed good protection (Keel *et al.*, 1989). Therefore, vermiculitic clay minerals seem to provide conditions of iron sufficiency for cyanogenesis and, in parallel, for efficient disease suppression by strain CHA0 (Keel *et al.*, 1989; Stutz *et al.*, 1989).

The influence of clay minerals on the production of secondary metabolites of the pseudomonads and on their disease suppressive capacity may explain, in part, why the same *Pseudomonas* strain protects plants in particular regions or fields but not in others.

## PROSPECTS

**Strain improvement.** The performance of biocontrol strains may be enhanced by the application of genetic techniques since the genes encoding relevant traits in the bacteria are now amenable to manipulation either at the level of regulation or by transferral to new hosts. However, this approach has to be handled with care since the bacterial determinants in question are not simple one-gene products and the impact on the environment cannot yet be evaluated.

A transposon carrying the genes coding for HCN production in *P. fluorescens* CHA0 was inserted into another *Pseudomonas* strain P3, which is naturally cyanide-negative and gives poor protection of tobacco from black root rot. The transgenic mutant synthesized HCN and had an improved ability to suppress disease but did not reach the suppressive capacity of strain CHA0 (Voisard *et al.*, 1989). Other examples of the transfer of bacterial genes involved in plant protection into other strains include the integration of the delta-endotoxin gene of *Bacillus thuringiensis* into the chromosome of root-associated *P. fluorescens* (Obukowicz *et al.*, 1987), and the introduction of a cosmid carrying genes that code for chitinase, from *Serratia marescens* into root-colonizing, fluorescent *Pseudomonas* spp. (Sundheim, this volume).

Production of the antibiotic oomycin A in *P. fluorescens* Hv37a was altered by fusing the *E. coli tac* promoter to a gene cluster essential for oomycin A biosynthesis (Gutterson, 1990). Oomycin A production in the rhizosphere and suppression of *Pythium* damping-off of cotton was then improved even under suboptimal conditions. Such an approach leads to the regulation of the production of active bacterial metabolites in a way that is less dependent on environmental conditions. In another approach a cosmid (pME3090) carrying a 22 kb insert of *P. fluorescens* CHA0 DNA enhanced, in a CHA0 background, the production of the antibiotics 2,4-diacetylphloroglucinol and pyoluteorin *in vitro* (Maurhofer *et al.*, submitted). The antibiotic-overproducing mutant CHA0/pME3090 improved the protection of cucumber from *Pythium ultimum*. In the absence of the pathogen, mutant CHA0/pME3090 had no negative influence on cucumber growth. In contrast, the same 22 kb amplification reduced the protection of sweet corn and cress from *Pythium*. Compared with wild-type CHA0, the antibiotic-overproducing mutant was clearly deleterious to plant growth in the absence of the pathogen, presumably because of a herbicidal effect of the two antibiotics on the plant. Thus, depending on the host-pathogen system, enhanced antibiotic production in *P. fluorescens*, can be both beneficial or deleterious (Haas *et al.*, 1991; Maurhofer *et al.*, submitted).

**Quality control.** An important objective is making bacterial strains more suitable for mass production. Strain CHA0, like other *Pseudomonas* strains is genetically unstable during storage in nutrient-rich media. The loss of tryptophan side-chain oxidase (TSO) in CHA0 can be used as a marker for its instability. TSO is a tryptophan catabolic enzyme initiating a pathway leading to indoleacetate (Oberhänsli *et al.*, 1991). After culturing CHA0 for eight days in nutrient yeast broth, up to 10% of the colonies had lost TSO activity. Spontaneous TSO-negative mutants differed from wild-type CHA0 in the production of secondary metabolites, such as HCN, pyoluteorin, and 2,4-diacetylphloroglucinol. The deficiences of the mutants correlated positively with a significant reduction in the suppression of take-all of wheat (Oberhänsli and Défago, 1991).

**Mixtures of beneficial microorganisms.** Another approach is the application of mixtures of beneficial microorganisms highly adapted to control specific diseases in specific environments. In recent field experiments Pierson and Weller (1991) found that treatment of wheat with combinations of certain *Pseudomonas* strains gave a better control of take-all of wheat than treatment with the individual strains alone. Use of four *Pseudomonas* strains in combination significantly increased grain yield by 20% as compared to the nontreated control. Lemanceau and Alabouvette (1991) and Fuchs and Défago (1991) report successful combinations of non-pathogenic *Fusarium oxysporum* strain Fo47 with specific fluorescent *Pseudomonas* strains to control *Fusarium* diseases of tomatoes in pot experiments and under commercial-type greenhouse conditions. These *Pseudomonas* strains, although having no disease suppressive effect on their own, significantly improved control by Fo47.

## CONCLUSION

Fundamental knowledge of the key traits of the bacteria involved in disease suppression and root colonization is necessary to understand the complex interactions between host, pathogen(s), bacterial antagonist and the biotic/abiotic environment and thus to open up possibilities for influencing this ecosystem and for disease control. Strain improvement by introduction or

amplification of specific genes, mixtures of beneficial microorganisms highly adapted to specific environments, development of adequate formulations and quality control, and the combination of biocontrol agent application with ecologically adapted crop management practices, may, help to improve the performance of biocontrol agents in disease control under field conditions.

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**ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF FLUORESCENT PSEUDOMONADS RHIZOBACTERIA.**

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Summary

Specific strains of *Pseudomonas fluorescens-putida* group rapidly colonize plant roots of several crops and cause significant yield increases. These beneficial rhizobacteries have also been described for their antagonistic activities against plant pathogenic fungi. The ability to produce siderophores seems to be connected with the enhanced plant growth and biological control.53 rizobacterial strains were isolated from wheat, onion, cabbadge, lettuce and rye-grass rhizosphere. We caracterized and identified 35 strains as *P. fluorescens*, 3 strains as *P. putida* and 15 as intermediate type *Pseudomonas fluorescens* (12 strains: gelatin+, trealose-; 3 strains: gelatin-, trealose+).
We also tested the siderophore production and the in vitro antagonistic activity against several phytopathogenic fungi: *Rhizoctonia solani* (RS1, RS2), *Fusarium* sp., *Fusarium oxysporum, Verticillium* sp., *Pyrenochaeta lycopersici* (Py-6b, Py-34, Py-39) and *Phytophthora cinnamomi.* These preliminary results allowed the selection of some strains of *P. fluorescens* with interest in biological control.

## Introduction

Fluorescent pseudomonads are prominent in the aerobic microflora of the rhizosphere of many plants. These bacteria have been reported to act as plant growth promoters (Digat, 1983). Several mechanisms have been proposed to explain these beneficial effects. One possibility lies in the antagonistic action against phytopathogenic microorganisms. This is related to the production of siderophores which are high-affinity Fe3+ specific chelating agents, therefore inhibiting deleterious microorganisms by depravation of iron (Gill & Warren, 1988).

## Material and Methods

### 1. Isolation and characterization

Roots of wheat, onion, lettuce, cabbadge and rye-grass plants were washed separately in sterile destillated water and suspensions were serially diluted and plated on King's medium B. Plates were incubated for 48 h at 27° C and then placed under UV light.
Fluorescent colonies were selected, isolated and their conventional characteristics tested (oxidase reaction, Gram reaction, liquefaction of gelatin, arginine dihydrolase, glucose and trealose utilization) to identify *Pseudomonas fluorescens-putida* and intermediate types (Digat & Gardan, 1987).

### 2. Siderophore production and antagonistic activity

A dense bacteria suspention was prepared and 5 l were placed on blue chrome azurol S medium (Klement et al., 1990). Plates were incubated for 24 h at 27° C. Siderophore production is showed by an orange halo around the colony. The colour turns from blue to orange when a strong chelator, e.g. a siderophore, removes the iron from the complex [ternary complex of chrome azurol S, iron (III) and hexadecyltrimethylammonium bromide].

Table 2- Comparision on antagonistic activity of 58 strains against 9 phytopathogenic fungi and bacteria siderophore production.

| Isolated | RS1 | RS2 | Fus. | F. ox. | Vert. | P. cin. | Py-6b | Py-34 | Py-39 | Sid. Pr. |
|---|---|---|---|---|---|---|---|---|---|---|
| cou 1 | ± | 0 | ± | ++ | ± | ++ | ± | ++ | ++ | II |
| cou2 | ± | 0 | 0 | ++ | ++ | ++ | +++ | 0 | ++ | I |
| cou3 | 0 | 0 | ± | ++ | + | +++ | + | + | ++ | I |
| cou4 | 0 | 0 | 0 | 0 | 0 | +++ | 0 | ± | ± | I |
| cou5 | 0 | + | + | ± | +++ | ++ | 0 | ++ | ++ | II |
| alf 1 | ± | ± | 0 | + | ± | ++ | ++ | + | + | II |
| alf 1.1 | ++ | ++ | ++ | ++ | ++ | • | ++ | +++ | ++ | II |
| alf 1.2 | • | ++ | +++ | +++ | +++ | • | +++ | ++ | • | II |
| alf 2 | ± | - | 0 | 0 | ± | ++ | + | + | + | I |
| alf 2.1 | • | ± | ++ | ++ | • | • | • | ++ | • | II |
| alf 2.2 | + | +++ | ++ | ++ | ++ | • | ± | ++ | ++ | II |
| alf 3.1 | • | ± | ++ | ++ | • | ++ | • | • | • | I |
| alf 3.2 | • | ++ | ++ | ++ | • | • | +++ | +++ | ++ | II |
| alf 4.1 | ± | ++ | ++ | ++ | + | • | ++ | ++ | + | II |
| alf 4.2 | • | ± | + | ± | +++ | ++ | +++ | +++ | • | II |
| alf 5.1 | ++ | + | ± | ± | ++ | • | ++ | ++ | ++ | II |
| alf 5.2 | • | ± | ++ | ++ | ++ | ++ | +++ | ++ | • | II |
| alf 6.1 | + | ± | ± | ± | + | • | ++ | ++ | ++ | II |
| alf 6.2 | + | ± | ++ | + | + | • | ++ | ++ | ++ | II |
| alf 6.3 | • | 0 | ± | ± | • | ++ | • | +++ | • | I |
| alf 7.2 | + | ± | ± | ± | ± | • | + | ++ | ++ | II |
| alf 8.1 | ++ | + | ++ | ++ | ++ | • | ++ | ++ | ++ | I |
| alf 9.1 | ± | + | ++ | ++ | ± | • | • | + | + | I |
| alf 10.1 | + | + | ++ | ± | ++ | • | ++ | ++ | ++ | II |
| alf 10.2 | • | ± | ++ | + | +++ | +++ | • | +++ | +++ | II |
| alf 11.1 | • | 0 | ++ | + | • | • | + | • | • | II |
| alf 13.1 | + | + | ++ | ± | ++ | • | ++ | ++ | ++ | II |
| alf 13.2 | + | ± | ± | ++ | ++ | • | ++ | + | + | II |
| alf 14 | + | + | ++ | ++ | + | ++ | + | ++ | ++ | II |
| alf 14.1 | • | 0 | + | ± | • | • | +++ | • | • | II |
| alf 15 | +++ | +++ | +++ | ++ | +++ | ++ | + | ++ | +++ | II |
| alf 15.1 | • | ± | ++ | +++ | • | • | • | • | • | II |
| alf 16.1 | + | ± | ++ | ++ | ++ | • | ++ | ++ | ++ | II |
| cebl | ++ | ++ | ++ | +++ | ++ | ++ | +++ | +++ | +++ | II |
| ceb3 | ± | ± | ++ | +++ | ++ | ++ | + | ++ | ++ | II |
| tr1 | ++ | ++ | ± | ++ | ++ | NT | NT | ++ | + | I |
| tr2 | ++ | + | ++ | ++ | ++ | NT | NT | ++ | ++ | I |
| tr3 | +++ | +++ | ++ | ++ | ++ | NT | NT | ++ | + | II |
| tr4 | ++ | + | ++ | ± | ++ | NT | NT | + | ++ | II |
| tr5 | +++ | +++ | ± | ± | ++ | NT | NT | • | + | II |
| tr6 | ++ | + | ± | ++ | ++ | NT | NT | + | + | I |
| tr7 | ++ | ± | ± | + | ++ | NT | NT | ++ | ++ | II |
| tr8 | ± | ± | ± | ± | ++ | NT | NT | + | • | I |
| tr9 | ++ | ++ | ++ | ++ | ++ | NT | NT | + | ++ | I |
| tr10 | +++ | +++ | ± | ± | + | NT | NT | • | + | I |
| z1 | +++ | +++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | II |
| z3 | +++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | II |
| z4 | ++ | ++ | ++ | ++ | ++ | +++ | ++ | +++ | ++ | I |
| z6 | ++ | ++ | ++ | ++ | ++ | ± | ++ | ++ | ++ | I |
| z12 | ++ | ++ | ++ | + | + | +++ | +++ | ++ | ± | II |
| z14 | +++ | +++ | ++ | ++ | +++ | +++ | ++ | +++ | +++ | II |
| z16 | +++ | ++ | ++ | ++ | +++ | +++ | ++ | +++ | +++ | II |
| z17 | +++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | II |
| z18 | ++ | 0 | ++ | ++ | ++ | • | ++ | + | +++ | II |
| z20 | +++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | II |
| z21 | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | II |
| z25 | ++ | +++ | ++ | ++ | ++ | +++ | ++ | +++ | +++ | I |
| z27 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | I |

0 Antagonism absent, the fungus grows over the bacteria; ± Weak antagonism, disappearing before 24 h; + Antagonism present up til 24 h; ++ Antagonism present between 24 - 48 h; +++ Strong antagonism during all the time; • Tested, positive antagonism but not classified according to the scale; NT not tested

I / II Halo of siderophore production after 24 h (ø ≤ 1.5 cm)/(ø > 1.5 cm)

RS1/2 = *Rhizoctonia solani* (tomato/cabbadge); Fus. = *Fusarium* sp.; F. ox. *F. oxysporum*; Vert .= *Verticillium* sp.; P. cin. = *Phytophthora cinnamomi*; Py = *Pyrenochaeta lycopersici*.

For the antagonistic test a medium of King+PDA (1:1) was prepared. A inoculer of 8 mm diameter of mycelium fungi was placed in the center of the plate and 4 spots of bacterial suspension (representing 4 strains of *Pseudomonas*) were placed around it. The distance and inoculation time of fungi/bacteria varies according with the growth rate of the fungi. These plates were incubated at 27° C and the first readings begun 48 h after bacteria inoculation and finish 2 days later.

## Results

In Table 1 the affiliation of the bacterial isolates to the *Pseudomonas fluorescens-putida* group is presented together with the host plant from which they were isolated. Siderophore production and antagonistic activity are presented on Table 2.

Table 1 - Source (hostplant) and affiliation of bacterial isolates to the *P. fluorescens-putida* group.

| | Cabbadge | Lettuce | Onion | Rye-grass | Wheat |
|---|---|---|---|---|---|
| *P. fluorescens* | cou1 - cou3 cou4 | alf 1 - alf 1.2 - alf 2 alf 2.1 - alf 2.2 - alf 3.2 alf 6.2 - alf 7.2 - alf 8.1 alf 9.1 - alf 11.1 - alf 13.2 alf 14 - alf 14.1 - alf 15 | ceb 1 | z1 - z3 - z12 z16 - z18 - z20 | tr1 - tr2 - tr3 - tr4 tr5 - tr6 - tr7 - tr8 tr9 - tr10 |
| *P. putida* | cou 5 | alf 4.2 - alf 6.3 | - | - | - |
| + - | cou 2 | alf 5.1 - alf 15.1 - alf 10.2 alf 6.1 - alf 16.1 - alf 4.1 alf 7.1 - alf 13.1 | ceb 3 | z4 - z6 - z14 z17 - z21 - z25 z27 | - |
| - + | - | alf 3.1- alf 10.1 - alf 5.2 | - | - | - |

Intermediate types: + - (gelatin + trealose -); - + (gelatin - trealose +)

## Discussion

The above results led us to conclude that a great number of the bacterial isolates showed good antagonism against the tested fungi as well as siderophorer production. In this preliminary work it was possible to select 13 strains (alf1.2, alf4.2, alf10.2, alf15, cou2, ceb1, tr3, z1, z3, z14, z17, z20, z27). They all showed strong antagonism against a large spectrum of the tested fungi.
In our future research the screening will be continued in order to obtain more strains. Also, the bacterial effects on plant growth will have to be tested in the laboratory as well as under field conditions in order to get a viable formulation of antagonistic inoculer and biofertilizers suitable for agricultural use on a large scale.

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# INHIBITION OF FUNGAL CELLULASE PECTINASE AND XYLANASE ACTIVITY BY PLANT GROWTH-PROMOTING FLUORESCENT PSEUDOMONAS SPP.

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Summary
The influence of metabolities of 14 plant-growth promoting and neutral fluorescent *Pseudomonas* spp. on cellulase, pectinase and xylanase activity was examined. The activity of cellulase was inhibited in the range from 29 to 100% by most of tested rhizobacteria strains. However, in the case of pectinase and xylanase the inhibitory activity was not so often observed. And the activity of pectinase and xylanase were inhibited in smaller range (12 - 60%). Three strains inhibited the activity of all tested enzymes, only. There were PGPR strains *P. fluorescens* B6/2 and *Pseudomonas* sp. PPs16 as well as NRB strain *Pseudomonas* sp. PPs96. Heat treatment by 20 min. did not decrease the inhibitory activity of sterile supernatants obtained from cultures of *P. fluorescens* B6/2 and *Pseudomonas* sp. PPs11. The inhibitory activity of strains PPs17 and PPs20 were destroyed in higher level by 20 min. heat-treatment complety by longer heating. The heat-stable proteinases could be anticipated in these cases. The ability of PGPR strains to inactivate the cell wall degrading enzymes of phytopathogenic fungi, could be taken into account as an additional mechanism explaining the phenomenon of biocontrol properties of fluorescent pseudomonads.

## Introduction

Extracellular plant cell wall degrading enzymes (CWDE) of soil-borne phythopathogenic fungi take part in the process of infection of plant tissues (Mullen and Bateman, 1975; Zalewska and Urbanek, 1981; Cooper, 1983). Several authors have discussed diverse mechanisms of interaction between beneficial fluorescent pseudomonads and different soil-borne pathogens. In pervious study Pietr (1987, 1990) reported the ability of two strains of *P. fluorescens* B6/2 and B6/4, to the inactivation of extracellular polysaccharide hydrolases of phytopathogenic Fusarium spp. He suggested that extracellular heat-stable proteinases could play some role in the inactivation of polysaccharide hydrolases of soil-borne fungi. As a continuation of the study , in this paper, we compared ability of different rhizosphere pseudomonads to inactivation of model fungal cell wall degrading enzymes.

## Materials and methods

### 1. Microorganisms

Fourtheen fluorescent pseudomonads were orginaly isolated from roots of field growing winter wheat, tomato, and cucumber. In this study were used seven pseudomonads siderophore producers (isolates denoted B54, DPs15, PPs11, PPs15, PPs16, PPs20, PPs21) and one pseudomonads antibiotic producer (isolate denoted 88f), which showed plant growth promoting activity. Plant growth promoting rhizobacteria siderophore producers *P. fluorescens* biotyp V strains B6/2 and B6/4 were described previously (Pietr, 1987, 1990). Additionally, four neutral rhizobacteria strains, were included into investigation *Pseudomonas*

spp. PPs96, Ps395, and PPs17 siderophore producers as well as Pseudomonas sp. Ps85 antibiotic producer.

2. Chemicals

Soluble carboxymethyl cellulose, pectin, and xylan were purchased from Loba-Chemie (Austria), Koch Light Lab. Ltd. (England), and Koch Light Lab. Ltd. (England), respectively. Others reagents were purchased from POCh (Poland). Mixture of cellulase, pectinase, and xylanase was prepared from Trichoderma viride by dr. D. Witkowska from Department of Technical Microbiology (Agricultural University of Wroclaw).

3. Preparation of supernatants

For production of extracellular metabolities the pure bacterial isolates were inoculated into King medium B solutions and after 72 hr the bacterial cells were centrifugated (4$^{o}$C, 3500xg, 30 min.). Such obtained supernatants were filtrated through sterile FlowPore D26 memebranes of pore size 0.20 $\mu$m (Sartorius, Germany). They were frozed at $-18^{o}$ for long term storage.

4. Enzyme assays

The activities of tested enzymes were assayed at 30$^{o}$C by incubating 0.5 mL (0.5 mg of crude preparate per 1 mL of 0.1 M citric buffer, pH 5.0) of a enzymes solution with 0.25 mL of a substrate (CMC, pectin or xylan 0.5 % solutions). The 0.5 mL of tested supernatants, diluted twice with citric buffer, were applied to the reaction mixtures. In control samples the sterile King B medium were used. The reaction was stopped by adding 2 mL of 1% zinc sulphate and 2 mL 0.06 N barium hydroxide and after centrifugation samples were analyzed for reducing sugars (glucose, galactose, xylose) by the Nelson method (Nelson, 1944). All deteminations were done in triplicate.

5. Thermostability studies

Four supernatants, which showed the strongest inhibitory activity against cellulase, were taken for this study. Effect of heating at 100$^{o}$C by 20 and 60 min. was determined. After heating sub-samples were taken for analyses as described above.

## Results and discussion

In several experiments the activity of cellulase, pectinase, and xylanase were significantly decreased due to presence of sterile supernatants of tested rhizosphere Pseudomonas spp. (Table 1). However, in few cases we observed a significant increase of the activity of tested enzymes. The activity of cellulase was inhibited in the range from 29 to 100% by all tested PGPR strains and two NRB strains. Among them, metabolities of three PGPR strains (B6/2, PPs11, PPs20) and one NRB strain (PPs17) inhibited the activity of cellulase at least by 81%. However, in the case of pectinase and xylanase the inhibitory activity was not so often observed. And the activity of pectinase and xylanase were inhibited in smaller range (12 - 60%), by four PGPR and two NRB strains, only. Three strains inhibited the activity of all tested enzymes. There were PGPR strains P. fluorescens B6/2 and Pseudomonas sp. PPs 16 as well as neutral rhizobacteria strain Pseudomonas sp. PPs96. Additionally, the inactivation of two tested CWD enzymes by compounds secreated by tested rhizobacteria was found in the most cases of PGPR strains. Ecepect for the strain PPs 96 all three NRB strains inhibited the activity of one enzyme, only. The observed phenomenon of the inactivation of fungal polysaccharide hydrolases by extracellular compounds secreated by rhizobacteria from the genus Pseudomonas was found to be quit often. Extracellular proteinases could be suggested to have a role in the inactivation of CWDE by rhizobacteria. Several strains of P. fluorescens are well known as producers of wide range of proteases with broad-spectrum of activity (Richardson, 1981; Stepaniak et al., 1982; Patel et al., 1986).

Table 1
The influence of sterile supernatants on the activity of cellulase, pectinase, and xylanase.

| Tested strains | Activity of enzymes (% of control) | | |
|---|---|---|---|
| | Cellulase | Pectinase | Xylanase |
| Control | 100 b* | 100 cd | 100 bc |
| | Plant growth promothing rhizobacteria | | |
| B 6/2 | 19 hi | 82 ef | 75 efg |
| B 6/4 | 33 fg | 106 c | 88 cde |
| B 54 | 26 gh | 84 ef | 161 a |
| DPs 15 | 56 d | 107 bc | 70 fg |
| FPs 11 | 8 j | 73 f | 152 a |
| PPs 15 | 43 e | 91 de | 73 efg |
| PPs 16 | 42 e | 41 g | 79 efg |
| PPs 20 | 0 k | 94 de | 85 cdef |
| PPs 21 | 71 c | 107 bc | 79 efg |
| 88f | 34 f | 165 a | 155 a |
| | Neutral rhizobacteria | | |
| PPs 17 | 18 i | 111 bc | 104 b |
| PPs 96 | 37 ef | 83 ef | 40 h |
| Ps 85 | 105 b | 119 b | 65 g |
| Ps 395 | 139 a | 51 g | 95 bcd |

* - values in the column followed by the same letter do not differ significantly (P = 0.05) according to Student t-test,

Table 2
Activity of celullase (μmoles of glucose released per min.) in the presence of sterile supernatants from selected pseudomonads after heat treatment.

| Objects, | Tested strain | | | | |
|---|---|---|---|---|---|
| | None | B 6/2 | PPs 11 | PPs 17 | PPs 20 |
| Control | 8.97 a* | 8.97 a | 8.97 a | 8.87 a | 8.87 a |
| Unheated supernatants | 8.97 a | 1.80 c | 2.73 c | 1.33 c | 1.60 c |
| Heat treatment 20 min. | 8.97 a | 0.97 d | 3.13 c | 5.30 b | 7.30 b |
| Heat treatment 60 min. | 8.97 a | 7.77 b | 5.00 b | 9.53 a | 8.57 ab |

* - values in the column followed by the same letter do not differ significantly (P=0.05) according to Student t-test,

Furthermore, observed increase of the activity of CWDE in the presence of some supernatants can be related to their own activity. The polysaccharide hydrolases activity of non-pathogenic rhizobacteria during theirs invasion into endorhizosphere were reported by Darbyshire and Greaves (1971) and Foster and Rovira (1976).
Results in Table 2 reported the further study with the four most active producers of the cellulase inhibitors. The heat-stability of these inhibitors

was investigated because in pervious study (Pietr, 1990) tested factors of P.fluorescens B6/2 and B6/4 were stable at 50°C. Heat treatment by 20 min. did not decrease the inhibitory activity of sterile supernatants obtained from cultures of P. fluorescens B6/2 and Pseudomonas sp. PPs11. However, 60 min. heat treatment caused significant dissolution of the inhibitory activity. The inhibitory activity of supernatants from strains PPs17 and PPs20 were destroyed in higher level by 20 min. heat treatment and completly by longer heating. Several pseudomonads excrete heat-stable proteases (Richardson, 1981; Stepaniak et al., 1982; Patel et al., 1986). The heat-stable proteinases with different resistance to heat treatment could be anticipated at least in these four investigated cases.
The enzymatic degradation of the CWDE of phytopathogenic fungi, could be taken into account as an additional mechanism explaining the phenomenon of biocontrol properties of fluorescent pseudomonads.

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# FLUORESCENT PSEUDOMONADS OCCURRING ON WHEAT SEEDS COMPARED TO PLANT GROWTH-PROMOTING RHIZOBACTERIA

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Summary
The microflora of seeds of four winter wheat cultivars was studied to find out details about bacteria and fungi occurring in this special environment. It was found that fluorescent pseudomonads frequently occurred on wheat seeds, especially in the early stage of ripening. Representative strains of these pseudomonads inhibited the growth of some Fusarium in vitro. The seed-pseudomonads were found to tolerate several fungicides which are commonly used for seed dressing. These properties of the seed-pseudomonads and their sensitivity to antibiotics were compared to several PGPR strains isolated from the rhizosphere of various crops in Hungary.

## Introduction

### 1. Non-pathogenic microflora of seeds

The surface of wheat seeds can be an advantegous environment for epiphytic microorganisms during the ripening. The seed is covered by the glume and by the palea, so it is protected from direct sunlight and from rapid drying out. The phytopathogenic microorganisms occurring on the seeds have been studied for many years. It is wellknown that many pathogens of wheat can be transmitted by seeds and there are fungi which may damage the seeds during the storage. On the contrary, the non-pathogenic microflora of seeds is hardly known - even less than other epiphytic microorganisms. The role and the importance of non--pathogenic seed-microorganisms is uncertain as well. It is questioned whether they are able to survive the storage and to colonize the seedling's rhizosphere. On the other hand the inoculation of seeds of different plants with fluorescent pseudomonads has been successful (Weller & Cook, 1983).

### 2. PGPR strains and their application for biological control of wheat diseases

The phenomenon of disease-suppressive soils is wellknown for many years. It means that some soil-borne diseases are depressed although the pathogens are present in the soil. This suppression may be caused by soil bacteria belonging to the genus *Pseudomonas.* They can rapidly colonize the rhizosphere of crops and, as a result may reduce diseases and increase the growth of host plants (Cook & Rovira, 1976). Based on their effect they are termed plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1980). Isolated from suppressive soils or from the rhizosphere they have been used for inoculation of a wide range of crops. The experiments resulted in more or less increase of the yield, and the severity of different soil-borne diseases was reduced. There are several factors which may explain the beneficial effect of PGPR. These are the rapid colonization of the rhizosphere, the production of siderophores (which bind iron(III)) and of antibiotics and plant growth-promoting substances (Schroth & Hancock, 1982). Many authors have proved that fluorescent pseudomonads inhibited the wheat-pathogen *Gaeumannomyces graminis* both in vitro and in vivo. Pseudomonads seemed to be effective against *Rhizoctonia* and *Phytium* root diseases of wheat. But there are only few reports on interactions between PGPR and other important wheat pathogens (e.g. Fusaria) (Alabouvette, 199o).

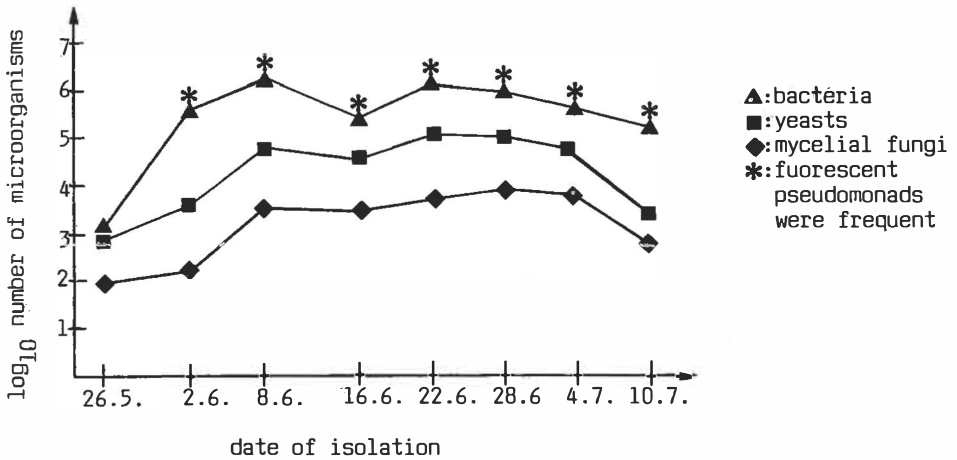
## Materials and methods

The microflora of wheat seeds was studied in two years. In 1987 seed samples derived from half a year and one and a half years storage or were collected from a field trial of the Agricultural University at Gödöllő in the stage of milk ripeness, wax ripeness and full ripeness. Each sample was taken from the same winter wheat cultivar Mv 8.
In 1989 samples of three winter wheat cultivars (GK Öthalom, Mv 10 and Bucsányi 20) were collected weekly from five different locations from the time of ear formation to full ripeness.
Seeds were taken out of the ears under sterile conditions (in laminar box using sterile forceps), and glume and palea were removed in order to examine the seeds only. 100 seeds were collected from each sample and were shaken in sterile water for 20 minutes.The enumeration of microorganisms was carried out by using dilution plate technique. The number of microorganisms was referred to one seed. The in vitro antagonistic effect of pseudomonads from seeds was studied on *Fusarium graminearum, F. culmorum, F. heterosporum, F. moniliforme, F. equiseti* and *F. acuminatum* (all strains isolated from wheat), whereas their effect on wheat seedlings was checked in pots using soil inoculation.
Response to fungicides of these bacteria was examined by amending agar plates with benomyl, triadimenol, mancozeb, carboxin, copper(III)oxychloride, hymexazole and thiophanate-methyl fungicides respectively. Antibiotic resistance was tested by putting antibiotic containing disks of filter paper on agar plates.

## Results

In 1987 the total number of microorganisms obtained on seeds showed a tendency to decrease during the ripening. Their number was about $10^7$/grain in milk ripeness, $5x10^5$/grain in wax ripeness and $10^5$/grain in full ripeness. The number of propagules was about $10^4$/grain after half a year of storage, and decreased to $10^3$/grain after one and a half year of storage. Gram-negative bacteria dominated in the microflora in all specimens. Fluorescent pseudomonads were present in a large number in milk ripeness stage but only a few remained later.In contrast to bacteria the number of fungi increased gradually during ripening and it reached it's maximum on stored seeds.
In 1989 the number of bacteria on samples increased rapidly first and reached a maximum of $1.5x10^6$/grain. After a second maximum there was a gradual decline in the number of bacteria. Yeasts and fungal mycelia reached their maximum significantly later (Fig. 1.).
The fluorescent pseudomonads made up a large proportion of the total bacteria, sometimes they were the only representatives of bacterial microflora. Since they were resembling PGPR strains isolated from the rhizosphere of several plants in Hungary, they were screened for antagonistic effect. One fifth of the isolates was found to inhibit the growth of several *Fusarium* spp.
The growth of seedlings treated with bacteria was measured and compared to the untreated control. Only one *Pseudomonas* strain resulted in significant increase in plant growth.
Each tested strain grew on media containing 10 mg/g of either benomyl, triadimenol, thiophanate-methyl or carboxin. On hymexazole, mancozeb and copper(III)-oxychloride however grew all strains at the concentration of 0.1 mg/g except two strains which were more sensitive to hymexazole and mancozeb. PGPR strains tested at the same time showed similar degree of resistance. The antibiotic resistance spectrum of both PGPR and seed-pseudomonads were very similar, too. All of them seemed to be resistant to cefalexin and colistin, almost all of them to chloramphenicol, half of them resisted streptomycin.

Figure 1.
Populations of bacteria, yeasts and mycelial fungi on wheat seeds




Conclusions

Because of their similarity to PGPR strains, the seed-pseudomonads are worth investigating further. Their origin and ability to colonize the rhizosphere are to be cleared up, as the background of their antibiosis and resistance to pesticides. In a preliminary experiment small plasmids (2 and 3 MD) were found in some of the strains, which might be used for future genetic manipulation of fluorescent Pseudomonas strains.

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# RELATIONSHIPS BETWEEN S 57 STREPTOMYCES SP. AND ROOTS AND ITS UTILIZATION IN THE IMPROVEMENT OF CROP PRODUCTION

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## Summary

This research has been carried out using S 57, a strain of *Streptomyces* sp. (NCIMB 40227) isolated from roots of Camellia. Morphological and physiological characteristics of S 57 useful for taxonomic descriptions and its *in vitro* activity against phytopathogenic fungi are reported.
The studies carried out by means of scanning electron microscopy led to interesting results about the relationships that S 57 develops with seeds and roots of different cultivated plants. It is pointed out that this micro-organism abundantly colonizes the seed coats and controls pathogenic and saprophytic mycoflora. The root cortical tissues are colonized too, and S 57 also grows into the cellular lumen.
*Streptomyces* sp. S 57 was employed in greenhouse trials, adding the micro-organism to the soil or using it to treat seeds. No negative effect on plant growth or root development was observed. On the contrary, in most cases the plants utilized in our trials were positively influenced, frequently showing improvement of crop production. The mechanisms in this process are at present being investigated, nevertheless both nutritional effects and protection against biotical and abiotical stresses can be proposed as being involved.

## Introduction

Presence and role of actinomycetes in rhizosphere have been widely studied, and some reports contain indications about the use of these bacteria as an agent for the biological control of plant diseases up to "Mycostop" commercial application (Lahdenperä et al., 1991), but as far as we know, only few cases of growth stimulation have been reported.
In the past years a lot of research has carried out in our Institute on the ecology of the actinomycetes in the rhizosphere; these studies evidenced the presence of endophytic streptomycetes within healthy plant roots (Quaroni et al., 1986).

## Results

### 1. S 57 characteristics.

*Streptomyces* sp. S 57 was isolated from surface sterilized roots of *Camellia japonica*.
Taxonomic characterization of S 57 was performed according to the latest edition of Bergey's manual of systematic bacteriology (Williams et al., 1989). The results of the 50 tests required for probabilistic identification, elaborated by the Matiden computer program, closely related S 57 to *Streptomyces rochei*, however there is a high standard error with regard to the taxonomic distance, indicating that S 57 is much more distant from the centroid than the average member of the cluster.
The strain was deposited at the National Collection of Industrial and Marine Bacteria (Aberdeen, U.K.) as NCIMB 40227.

2. Micromorphological studies.

The observations were carried out by means of scanning electron microscopy (SEM) on seeds and roots treated with a suspension of spores of S 57 and on roots of plants grown in soil with the actinomycete added. Samples of different plant species (e.g. tomato, basil, cyclamen) were prepared by two techniques, differing in their stages and results: the first of them, called Freeze Drying (FD), is very simple and doesn't use any chemical treatment, while the second, named Critical Point Drying (CPD), is more complex and can give rise to some artefacts, due to the action of the solvents that remove the slime covering surfaces and cell walls of plants and microorganisms (Petrolini et al., 1986).

SEM observations showed that S 57 is able to grow on a wide range of plants and develops and sporulates on seed surfaces, without any problem about germination: on the contrary treated seeds often had a better germination than the untreated.

To investigate the situation inside the roots some samples were cryofractured and FD prepared. Sometimes it was possible to see hyphae and spore chains developed by S 57 in the cortical cells; the inside origin of these structures is confirmed by the presence of the slime surrounding and the partial coating of them.

In samples prepared without incubation it is very difficult and time consumig to find the microorganism hyphae which are small sized, few and often coated and hidden by the mucilaginous layer. To make the investigation easier some of the specimens were incubated in a moist chamber; the incubation promoted the development of the streptomycete, leading to an abundant growth of S 57. In such samples it was possible to see well developed spore chains.

3. In vitro activities.

Streptomyces sp. S 57 inhibited in vitro the growth of Fusarium spp., Rhizoctonia solani, Thielaviopsis spp., Cylindrocarpon spp., Trichoderma spp. and Geotrichum spp. Particular attention must be paid to the anti-Fusarium activity that is strong and spread to all the tested strains.

The activity against phytopathogenic fungi was also studied using Zea mays seeds, naturally infected by Fusarium sp. The seeds, wetted with a suspension of spores were incubated in germinators for 10 days. Some remarks must be made on these results:

- In S 57 treated seeds the germination increased from 80% to 92%.
- The presence of Fusarium sp. colonies on the surface of treated seeds decreased from 88% in the control group to 24% in the treated one.
- The percentage of plants showing secondary roots increased from 50% in the control to 73.91% in the treated sample.

4. Greenhouse trials.

S 57 was tested on 18 different cultivated species. As a method of inoculation sometimes seed coating was used and other times direct addition of S 57 to the soil by means of a spore suspension.

Thirteen plant species have been tested in the greenhouse with positive results from a commercial point of view, no negative effects were observed in the other five tested species. The results obtained with Rhododendron hirsutum and Cyclamen persicum will be displayed as examples.

In Rhododendron hirsutum the evaluation was based on plant diameter, whose increase leads to different commercial value. Two classes (diameter from 20 to 25 cm and from 25 to 30 cm) were considered. Only 28% of the control plants fell in the second, more valuable, class while this treatment gave rise to 50% of large plants, that also had a significant increase in the number of flowers per plant. These differences mean an increase of about 7% in the commercial

value.
Results obtained with cyclamen at the time of sale are recorded in the table.

| Cultivar | % flowering plants | | flowers/plant | |
|---|---|---|---|---|
| | control | S 57 | control | S 57 |
| Tosca | 88 | 100 | 5.02 | 6.58 |
| Orpheus | 74 | 88 | 3.06 | 3.41 |
| Oberon | 84 | 98 | 4.59 | 4.60* |
| Ophelia | 100 | 100* | 5 | 7.13 |

* Only these values are not significantly different at P=0.05

Discussion

Streptomyces sp. S 57 is able to grow in the inner cortical tissues of many plant species, and the role that it plays on growth and productivity of various species suggests that its host range would be wide (Sardi, 1991). These results suggest the existence of more or less complex relationships between the microorganism and the roots and the aim of future work is also to clarify them.
At present, we have not yet completely verified the mechanisms of action of S 57. It could interact with plant growth either by influencing nutrient uptake or by the in loco production of secondary metabolites.
Also the protection that S 57 could give against soil-borne pathogens would be very interesting and the topic is presently been studied.

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# BIOLOGICAL CONTROL OF SEED ROT AND DAMPING-OFF OF SUGAR BEET WITH MICROBIAL ANTAGONISTS (Abstract)

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The aim of our studies was to control seed rot and damping-off of sugar beet by application of microbial antagonists to the seed.
102 bacteria and actinomycetes were isolated from soil suppressing the in vitro growth of *Pythium ultimum* (20°C). The most efficient antagonists were applied to sugar beet seed as a suspension. The effect on disease incidence was evaluated in greenhouse experiments (12° and 18°C) using soil artificially infested with *Pythium ultimum*. The treatment with antagonists increased seedling emergence and resulted in a significant decrease of disease severity up to 84% by means of plant fresh weight. The antagonistic activity did not depend on the cultivar used.
Furthermore, *Pythium irregulare* and *Pythium mamillatum* participating in damping-off of sugar beet were successfully controlled by these antagonists. The growth of *Phoma betae*, *Rhizoctonia solani* f. sp. *betae* and *Fusarium oxysporum* f. sp. *betae* was successfully inhibited in vitro. None of the micro-organisms isolated effected *Aphanomyces cochlioides*.
The most efficient antagonists were identified as three isolates of *Pseudomonas fluorescens*, two isolates of *Pseudomonas maltophilia* and three *Streptomyces* spp..
The most effective suppression of *Pythium ultimum* was obtained by a combined application of *Pseudomonas fluorescens* B5 and *Streptomyces* sp. A102.
Beside the evaluation of disease incidence in the greenhouse a latent root infection with *Pythium ultimum* was studied by light-microscopy of stained roots. *Pseudomonas maltophilia*

isolates were most effective in protection against a latent infection.
Investigations on the antagonistic mechanisms showed *Pseudomonas fluorescens* isolates to produce antibiotics, siderophores and HCN.
An antibiotic from *Streptomyces* sp. A102 was partially purified; thin layer chromatography revealed a major band corresponding to oligomycin.
In order to improve application and survival, the antagonistic isolates were included in the process of seed pelleting (in cooperation with the Kleinwanzlebener Saatzucht AG, Einbeck). In this way the microbial activity was assured for several weeks. The pelleted seed was sown in naturally infested soil in the field. The pelleting of seed with microbial antagonists resulted in an up to 13% higher field-emergence.

# INTERACTION OF PSEUDOMONAS FLUORESCENS WITH PYTHIUM SPECIES.

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## Summary
Two strains of *Pseudomonas fluorescens* were tested *in vitro* for their effect on *Pythium ultimum* and *P. aphanidermatum*. On nutrient poor agar media, the *P. fluorescens* strains were overgrown by the pathogen. On agar media with a higher nutrient level the bacterial strains overgrew the fungal colonies. Antagonism was evident on media such as soil extract agar. When the bacteria were applied as seed dressings to pea seeds germinated on agar *in vitro*, protection was ineffective if the nutrient status of the medium was high. Preliminary studies enumerating *Pythium* propagules from soil indicate that the pathogen population may rise from 300 to 1350 propagules/g of soil within 72 hours.

## Introduction
Seeds of many important crop plants are vulnerable to attack by *Pythium* species. These fungal pathogens can survive as saprophytes in the soil becoming parasitic in the presence of living host tissue (Webster, 1980). Biological control of *Pythium* species may be feasible, since the period of host susceptibility is relatively short, lasting only a few days while the seedlings emerge. Therefore, a biological control agent (BCA) need only remain active for a short period.

Since the seed and root zones of plants are nutrient rich, most interactions between the pathogen and potential antagonist will occur in these regions. To effectively suppress disease and protect the host plant, it is necessary to understand the behaviour of micro-organisms in the spermosphere and rhizosphere.

## Materials and Methods
The method of Stanghellini and Hancock (1970) was used to estimate the number of *P. ultimum* propagules in loam soil. Infested loam was produced by inoculating autoclaved loam with actively growing mycelium from a *Pythium* sandflask. Controls consisted of uninoculated sterile loam. This experiment was repeated four times and was also performed once with non-sterile loam.

Two strains of *Pseudomonas fluorescens*, coded Pf/01 and Pf/02, were selected in preliminary screens based on protection of seedlings against pre-emergence damping-off by *Pythium*. Growth and activity of the BCA strains and *Pythium* species were examined *in vitro*. Plates of agar media of varying nutrient status were inoculated with *P. ultimum* or *P. aphanidermatum* with or without Pf/01 or Pf/02. Plates were incubated at 20°C and examined after 2 and 7 days for fungal colony diameter, growth of the BCA and antagonism. This experiment was repeated with the BCA strains applied as seed dressings to surface disinfested pea seeds. Controls utilised the pathogen alone, or unprotected pea seeds.

## Results

Enumeration of P. ultimum indicated that in previously sterile loam the population of the fungus rose from 300 to 1350 propagules/g of soil within 72 hours. In non-sterile loam, the population rose from 200 to 4000 propagules/g within the same time period (Figure 1).

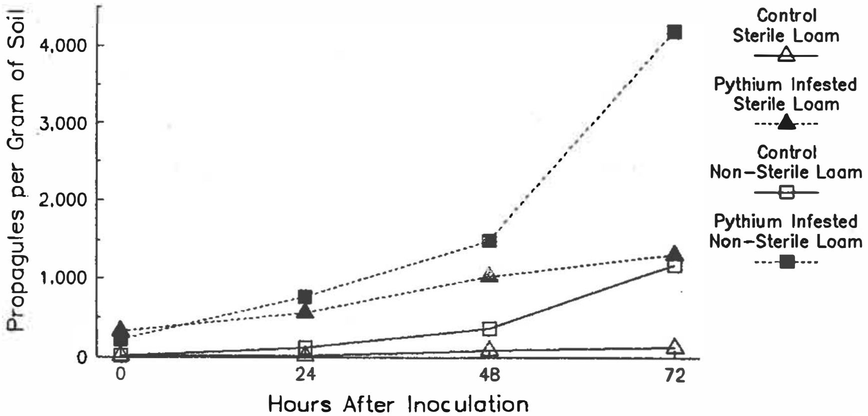


Figure 1 – Loam was used in all cases. Sterile Loam was produced by autoclaving. Pythium was added as actively growing mycelium.

Both strains of P. fluorescens (Pf/01 and Pf/02) showed antagonism in vitro to P. ultimum and P. aphanidermatum, initially as a zone of inhibition around the bacterial colony, followed, in some cases, by complete overgrowth of the pathogen by the BCA. The extent of the antagonism depended on the nutrient level in the agar media. On low nutrient media, the BCA strains grew poorly and failed to compete with the fungus. On nutrient rich media the pathogen grew vigorously and overcame the antagonism. On more natural media, such as soil extract agar (SEA), the BCA strains outgrew and actively antagonised the pathogen.

The nutrient level of the substrate was also important when the bacteria were applied as seed dressings. Again, protection was not apparent on either nutrient poor or nutrient rich media. On substrates such as SEA, where the seeds were protected, there was a distinct zone around the seeds where growth of the pathogen was sparse.

Once the radicle had emerged then the seed, protected or unprotected, was no longer vulnerable to attack and did not subsequently become colonised.

## Discussion

*Pythium* species are very efficient colonisers and can quickly build up a large inoculum potential in the soil. Enumeration of *P. ultimum* propagules in autoclaved and re-inoculated soil indicates that a 4-fold increase is possible within 72 hours. When non-autoclaved soil is used, a 20-fold increase may be possible. The reason for this is unclear. It is possible that autoclaving may alter the soil structure and/or nutrient status, thus making it less suitable for growth of the fungus.

The biological control effect of the *Pseudomonas* strains used in this study appears to be linked, at least *in vitro*, to nutrient status. On nutrient poor media, the BCA strains do not grow and therefore do not have an antagonistic effect. If applied as a seed dressing on nutrient poor media, some protection is observed, since the bacteria can utilise seed exudates. Protection is not fully effective as the BCAs cannot grow away from the seeds and the antagonistic effect is limited to a narrow zone. At very high nutrient levels, the pathogen can outcompete the BCAs and colonise the seed. However, on SEA the BCA strains appear able to protect the seed during the vulnerable period of emergence. This is important, because SEA is nearer to actual soil nutrient status than the very rich or nutrient poor media on which protection was limited.

By examining the interactions of *Pythium* species and BCA strains around seeds a greater understanding of this system should be achieved. Combined with studies on the population dynamics of the pathogen and bacteria in soil, this will enable optimum use of these potential biological control agents.

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## BIOLOGICAL CONTROL OF DAMPING-OFF IN *PINUS SYLVESTRIS* L. WITH BACTERIAL ANTAGONISTS

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### INTRODUCTION

Culture of young coniferous trees, especially *Pinus sylvestris* L. is frequently accompanied by damping-off and root rot of seedlings. The disease is evident in greenhouse and outdoor cultures in forest nurseries and caused by several genera and species of soil-borne fungi like *Pythium spp., Fusarium spp., Rhizoctonia solani, Cylindrocarpon radicicola, Botrytis cinera, Phytophthora cactorum, Alternaria spp.*(ENEBAK et al.1990).
The course of the disease can be divided into to stages: Pre-emergence and post emergence infestation. The first is characterized by a decreased number of germinated and emerged plants. Post-emergence infestation is distinguished by browning and rotting of the hypocotyls connected with rotting roots and the collapse of the plants followed by their death (HUANG & KUHLMAN 1989).
The disease appears irregularly dependent on environmental conditions such as temperature, moisture status of the substrate and air and the composition of the substrate. At the present time treatment of the soil with concentrations of fungicides is the common method used against damping-off.
The aim of this study was to control damping-off in *Pinus* seedlings by the application of microbial antagonists as an alternative to the treatment of soils with fungicides.

### MATERIAL and METHODS

Infested pine seedlings were collected from some greenhouse experiments of the Institute of Forest Tree Breeding Waldsieversdorf. Seedlings with symptoms of the disease on the hypocotyls and roots were used for isolation of pathogens. After surface sterilization small pieces of roots and shoots were incubated 10 days ( from the 4th day with UV-light) by 25°C on malt agar (2%). The growing fungi were carried over on water agar and determined.
The fungal isolates were tested *in vitro* on malt agar (pH 6,0) against several bacterial strains with known antagonistic activity from the culture collection of Research Centre of Soil Fertility Müncheberg. Strains which acted positively were used in pot experiments. The following strains were selected: an antibiotic-producing *Pseudomonas* isolate (strain 17), a cytokinin-producing *Pseudomonas putida* (strain 27), an auxin-producing *Pseudomonas*

*putida* (strain 421), an antibiotic- producing *Bacillus subtilis*-strain. All pseudomonads produced fluorescent siderophores.
Bacteria were cultured on PY-medium (10g proteol. pepton, 3g yeast extract per 1l aq.dest.) for 24 h by 25°C, washed twice with 0,1m $MgSO_4$-solution and tap water and applied directly. Plants (1g seeds per pot) were cultured in the greenhouse at 25°C and >80% air humidity in pots with substrate containing sand, peat, and compost in properties of 3:1:1. The antagonists were applied in different ways: 1) Pre-emergence treatment by dipping the seeds into bacterial suspension with density of $10^8$cfu/ml; 2) post-emergence treatment by spraying of the bacterial suspension with the same density on soil surface after emergence of pine seedlings. Treatment of soil with Ridomil-Zineb (Metalaxyl) at a concentration of 10g/1kg of substrate was used as the control. After 14 days the emerged seedlings were counted, on week later the number of healthy plants and further 14 days number of dead pine plants was determined.

## RESULTS and DISCUSSION

The following soil fungi were isolated from roots and hypocotyls of damaged pine seedlings:
*Fusarium oxysporum, F.redolens,F.solani, Cylindrocarpon destructans,Alternaria sp., Rhizoctonia solani, Pythium irregulare,P.ultimum.* The most detrimental fungi were *F.oxysporum,R.solani* and *P.ultimum*. All fungi were inhibited *in vitro* by *Pseudomonas*-strains and *Bacillus subtilis*, but the inhibition zones were variable. *R.solani* and *P.ultimum* were mostly inhibited by *B.subtilis* and the antibiotic- producing *Pseudomonas*strain.
The experiments *in vivo* confirm the possibility to control damping-off in pine with the bacterial antagonists (tab.1). Treatment of seeds with antagonistic *Pseudomonas*-strains and *B.subtilis* with exception of the auxin-producing strain 421 showed an increase in the number of emerged pine plants. The effect of *B.subtilis* by this method of application was only slight. The number of healthy plants was increased by all treatments except the auxin-producing strain.
Following soil treatment with bacteria similar results were obtained. All fluorescent *Pseudomonas*- strains and *B.subtilis* controlled the soil-borne pathogenic fungi to the same extend as the fungicide. Most efficiency showed isolates 17 (antibiotic-producing) and 27(cytokinin-producing) in their influence on number of healthy seedlings and later on number of collapsed plants. In some cases like *B.subtilis* and *P.putida* strain 421 were no difference on the number of collapsed seedlings between the two methods of applications.
It seems that antagonists producing antibiotics in the speremathosphere or in the rhizosphere are most efficient against fungi causing damping-off. Stimulation of germination and the promotion of cell division at the growing roots by cytokinin-like compounds is a possible mechanism of disease control.

table 1:

**Influence of treatment of *Pinus sylvestris* with antagonistic bacteria on emergence, number of healthy, and collapsed seedlings** (relative to non-treated control)

| treatment | emergence | healthy seedlings | collapsed seedlings[1] |
|---|---|---|---|
| **without** (control) | 100 | 100 | 42,0 |
| **Ridomil-Zineb** (fungicide control) | 113 | 148 | 16,0 |
| **Pseudomonas sp.strain 17** seed treatment | 112 | 135 | 22,0 |
| **Pseudomonas sp.strain 17** soil treatment | - | 161 | 2,1 |
| **P.putida strain 27** seed treatment | 114 | 161 | 24,0 |
| **P.putida strain 27** soil treatment | - | 162 | 8,0 |
| **P.putida strain 421** seed treatment | 84 | 105 | 15,5 |
| **P.putida strain 421** soil treatment | - | 127 | 19,1 |
| **Bacillus subtilis** seed treatment | 106 | 138 | 11,3 |
| **Bacillus subtilis** soil treatment | - | 135 | 14,4 |

[1] related to number of healthy plants after 3 weeks

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# BIOCONTROL OF COTTON SEEDLING DISEASE IN PASTEURIZED AND NATURAL FIELD SOIL BY A PSEUDOMONAS STRAIN.

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## Summary

Natural and pasteurized field soil were artificially infested with Rhizoctonia solani. A Pseudomonas strain suppressed cotton seedling disease in both soils although the natural field soil in addition was infested with indigenous pathogenic Pythium. The problems and advantages arising from the use of both test systems for testing biological control agents were discussed.

## Introduction

Rhizoctonia solani Kuehn and Pythium ultimum Trow are the major pathogens in the seedling disease complex of cotton (Watkins, 1981). They occur in all cotton growing areas and can cause considerable losses. The symptoms vary depending on the developing stage of the host and the environmental conditions from seed decay, seedling damping-off to seedling stem canker. Despite long lasting efforts to develop effective control measures, especially post-emergence damping-off is still considered a major problem in cotton production.
More recently, soil microorganisms like rhizobacteria were evaluated by some researchers for their potential to control cotton seedling diseases (Currier et al.,1988; Howell and Stipanovic, 1979; Howell and Stipanovic, 1980; Nelson, 1988). However, the selection of an appropriate screening system for testing bacterial isolates for their biocontrol potential in the greenhouse is still controversial.
Our objective in the present study was to demonstrate the biocontrol efficacy of a Pseudomonas strain in cotton against post-emergence damping-off in a greenhouse assay with pasteurized and natural field soil respectively.

## Materials and methods

Plastic pots (280mL) were filled with either natural or pasteurized, but recolonized soil and cotton seeds were sown 1 to 1.5 cm deep (5 seeds per pot). The soil was infested with R. solani according to a previously described method (Kempf et al., 1991) which ensured almost exclusively post-emergence damping-off. Two alginate pellets each encapsulating 2.5mg of Rhizoctonia millet powder were added to the center of the pot 1 cm below the surface, and then drenched with 20mL of a bacterial suspension or pencycuron or/and metalaxyl respectively resulting in approx. $2 \cdot 10^7$ cfu/mL soil or 20ppm of each fungicide respectively. After 19 days the disease incidence was evaluated by rating each plant on a scale from 0 to 3 (0 = healthy, white hypocotyl; 1 = slightly infected, hypocotyl partly with brown lesions; 2 = heavily infected, hypocotyl completely rotted; 3 = dead plant, rotted, wilted, dried up; or not emerged). The mean ratings from 10 pots per treatment were averaged.

## Results and discussion

R. solani added to pasteurized soil resulted in a disease index of 2.6 to 2.8 (Tab.1). The Pseudomonas strain reduced the disease incidence to the level of the non-infested control. In the natural field soil cotton plants suffered from indigeneous pathogens, and a disease index of around 2.0 was calculated. Adding artificial inoculum of R. solani resulted in an increased average rating of 2.4 to 2.9. A reduced disease incidence (rating 1.1 to 1.7) was observed after the application of the biocontrol strain in natural soil with or without an artificial inoculum. Therefore, the bacterium was able to reduce the disease producing activity of the naturally occurring pathogen/s, and furthermore managed to completely control R. solani. Metalaxyl reduced the disease incidence in the natural field soil to the level of the pasteurized non-infested control which indicates the presence of indigeneous oomycetes, most probably Pythium ultimum.
When working with natural field soils one might encounter certain difficulties. The presence of indigenous pathogens could result in unwanted disease and highly variable results. Furthermore, biological control often is less efficient in natural field soil than in sterilized and pasteurized soil due to inhibitory effects of the indigenous microflora against the biocontrol agent (competition, antibiosis, parasitism). On the other hand, the ability to compete with a multitude of other soil microorganisms for limited resources is a major selection factor in the screening for potential biocontrol agents. Traits which ensure competitiveness (i.e. production of antibiotics) are energy consuming and under gnotobiotic conditions not advantageous. Even pasteurization has a profound impact on the biological buffering capacity of the soil (Merrimen and Russell, 1990). However, in practical terms one has to compromise between results with good reproducibility and low variability on one hand and conditions close to the field on the other. Therefore, we consider the use of pasteurized, but recolonized field soil as the best choice for our greenhouse assays.

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**Tab. 1** Suppression of seedling disease in natural and pasteurized field soil (disease index 0=healthy to 3=dead, mean ±sd).

| treatments | | exp. #1 | exp. #2 | exp. #3 | exp. #4 |
|---|---|---|---|---|---|
| **natural soil :** | | | | | |
| no treatment | / -Rs | 2.2 (±0.5) | 1.9 (±0.9) | 2.2 (±0.4) | 2.0 (±0.5) |
| no treatment | / +Rs | | 2.9 (±0.2) | 2.4 (±0.5) | 2.5 (±0.3) |
| bacterium | / -Rs | | 1.6 (±0.6) | 1.6 (±0.6) | 1.7 (±0.8) |
| bacterium | / +Rs | | | 1.7 (±0.5) | 1.1 (±0.5) |
| pencycuron | / -Rs | | | 1.9 (±0.5) | 2.3 (±0.6) |
| pencycuron | / +Rs | | | 1.9 (±0.4) | 2.0 (±0.5) |
| metalaxyl | / -Rs | | | 0.9 (±0.4) | 0.6 (±0.3) |
| pencyc.+metal. | / -Rs | | | 0.7 (±0.4) | 0.6 (±0.7) |
| **pasteurized soil :** | | | | | |
| no treatment | / -Rs | 0.5 (±0.5) | 0.4 (±0.3) | [1.6 (±0.2)] | 0.6 (±0.7) |
| no treatment | / +Rs | 2.8 (±0.2) | 2.6 (±0.9) | 2.7 (±0.3) | 2.8 (±0.1) |
| bacterium | / +Rs | 0.5 (±0.2) | 0.4 (±0.4) | | |
| pencycuron | / +Rs | 0.9 (±0.7) | 0.5 (±0.4) | | |

BIOLOGICAL CONTROL OF GERBERA WILT ON ROCKWOOL

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Summary
Gerbera wilt caused by Fusarium oxysporum f.sp. gerberae is a serious disease especially on micropropagated plants. Mycostop, a recently commercialized biofungicide based on a streptomycete strain, was tested in the control of gerbera wilt using artificially infested rockwool as a growing substrate. Mycostop was applied by soaking the rockwool blocks and/or by monthly spraying of the growing substrate. The results indicate that Mycostop is efficacious even in an inorganic substrate. In comparison with the untreated control, the additional flower yield ranged from 12% to 33% depending on the variety. Although the efficacy of Mycostop was proved, further studies are still required to establish the correct application doses and optimal timing of the sprayings.

Introduction

Fusarium oxysporum f.sp. gerberae is a serious wilt causing pathogen on gerbera. Micropropagated plants cultivated on rockwool are especially susceptible. Young tissue-cultured plants can often even die by the attack of Fusarium, whereas older gerberas usually survive, although their yield cannot compete with that of healthy gerberas. Chemical control with benomyl for instance, has not been sufficiently effective; resistant strains of the pathogen develop quickly and retarded plant growth has frequently been observed in connection with chemical fungicides.

Since Mycostop, a biofungicide based on Streptomyces sp., has been successfully used to protect carnation against Fusarium wilt, it seemed worthwhile to try it also on gerbera. The second aim of this preliminary study was to test whether the biocontrol agent can be active in an inorganic substrate, such as rockwool. So far, the efficacy of Mycostop has been demonstrated mainly in peat and soil, where organic material is available to promote the growth of Streptomyces.

Material and methods

This paper presents two preliminary greenhouse trials on the control of Fusarium wilt of gerbera on rockwool. These experiments were carried out in Kemira Oy Espoo Research Centre during 1990-91. The first trial included four cut gerbera (Gerbera x cantebrigiensis) varieties: Fame, Macho, Pacific and Party, 8 plants per variety. In this trial all rockwool blocks were artificially inoculated with F. oxysporum; half of the plants of each cultivar were treated with Mycostop and half were untreated control plants. The second trial was based only on one variety, Party. The test plants were grouped into four treatments of 32 plants: (1) healthy control, (2) infected control and (3-4) Mycostop treatments; treatments 2, 3 and 4 were artificially inoculated.

The artificial inoculation was carried out immediately after transplanting by introducing the aqueous suspension of F. oxysporum to the rockwool blocks. The inoculum density was $10^4$ cfu per plant. In the four-variety experiment only one mode of applying Mycostop was tested, viz. monthly spraying with 0.1% aqueous suspension, 1-2 ml per plant. In the single-variety trial two different application methods were tested: (a) soaking the rockwool blocks in fertilizer liquid with a 0.01% Mycostop content before planting and monthly spraying with 0.1% suspension, and (b) monthly spraying with 0.1% suspension only. In both trials, the first application was carried out on the day of transplantation.

Both the first quality grade and total flower yields were counted during the course of the experiments. The harvest period was four months in the four-variety trial and seven months in the single-variety trial. Stem lengths and flower diameters were also recorded. At the end of the growing period, the fresh weight of the stand was measured, root health was evaluated and the occurrence of *Streptomyces* in the rhizosphere and the adjacent rockwool was analyzed.

## Results

Mycostop treatments reduced the disease incidence and increased the flower yield in both trials. However, the first experiment was carried out on such a small scale (4 plants/treatment/variety) that the differences were not statistically significant. In contrast, the second trial (32 plants per treatment) demonstrated a significant yield increase. The additional flower yield in the four-variety test, in comparison with the control, ranged from 12% to 32% depending on the susceptibility of the cultivar (Fig. 1a). In the single-variety trial, the yield was brought to the same level or even higher than that of the non-inoculated plants (healthy control) by using Mycostop (Table 1). In addition to the yield increase certain quality properties were improved owing to the application of Mycostop; in gerbera varieties very susceptible to the disease, Mycostop sprayings increased stem elongation, the diameter of the flowers and fresh weight of the stands (Fig. 1b).

Table 1. Relative monthly flower yield of gerbera (cv. Party) after Mycostop treatments on rockwool.

| Treatment | Relative flower yield | | |
|---|---|---|---|
| | August | October | December |
| Healthy control | 100 | 100 | 100 |
| Infected control* | 80 | 82 | 77 |
| Mycostop spraying* | 95 | 110 | 117 |
| Mycostop soaking and spraying* | 79 | 97 | 100 |

* Artificially infected with *F. oxysporum*

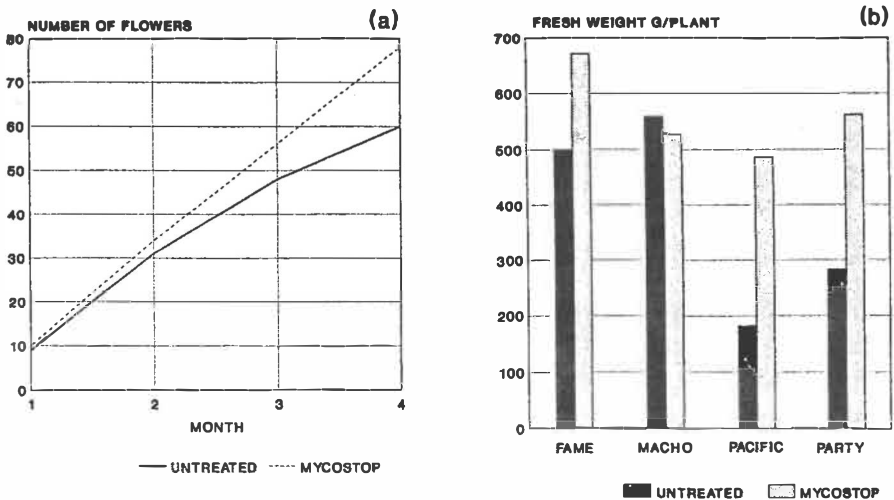


Fig. 1. Effect of Mycostop spraying in the four-variety gerbera test on rockwool artificially inoculated with *F. oxysporum*: (a) cumulative flower yield of the Pacific variety, (b) fresh weight of the stands.

## Discussion

As protection against *Fusarium* wilt of gerbera, Mycostop biofungicide provides an effective alternative to chemical fungicides also in an inorganic substrate, namely rockwool. Until now, this bioagent had mainly been tested in peat or soil, where organic material supports the growth of *Streptomyces.*

Mycostop treatments reduced wilt disease and consequently increased the flower production of gerbera. These results are similar to those obtained with Mycostop in the control of *Fusarium* on carnation grown in peat (Lahdenperä 1987). The higher yield of gerbera, rising even above that of healthy control, may be an indication of some stimulative factor involved.

In these preliminary trials, monthly spraying of rockwool blocks proved to be an effective treatment method, but the optimal mode of application is still being investigated. Delivery of the microbe through the irrigation system, for instance, could be of great help in treating stands of plants in large-scale greenhouse production. Studies are also needed to determine whether a longer interval between treatments could render sufficient results; on carnation in peat, the recommended treatment interval is 1-3 months.

In an earlier study on cucumber (Tahvonen, Avikainen & Lahdenperä 1991), an equal amount of Mycostop per plant was applied both in peat and rockwool cultivations. The whole surface of the peat was sprayed with Mycostop suspension, whereas in rockwool only the stem base was treated. This method resulted in an approximately 50 times higher local concentration and a lower yield in the rockwool cultivation at the beginning of the growing period. This is suggestive of the same phenomenon that was observed in the gerbera test where the rockwool blocks were soaked in Mycostop suspension: additional yield was obtained with Mycostop spraying alone already at the beginning of the harvest period, but no yield increase was achieved during the first three months when the soaking treatment was also used.

On the basis of the microbial analyses, *Streptomyces* seems to survive in rockwool, though no population growth was detected. While testing the stability of streptomycetes in rockwool, these microbes were isolated in amounts of $10^4$-$10^5$ cfu/g six weeks after the rockwool cubes had been soaked in Mycostop suspension. Immediately after Mycostop soaking, the number of colony-forming units amounted to $10^5$ cfu/g. Although streptomycetes are typical soil organisms, they are also known to be able to survive in exceptional surroundings, e.g. in water and plant debris. The present studies suggest that these actinomycetes can even act as active antagonists in an inorganic substrate as well, probably due to their ability to utilize root exudates and sloughed root cells.

Further trials are now being carried out both in experimental and commercial greenhouses. If they produce similarly positive results, the use of Mycostop can also be highly recommended in rockwool. Furthermore, the use of this biofungicide in the control of gerbera wilt, regardless of the type of substrate, offers a welcome alternative, since new methods for controlling root pathogens are needed.

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BIOCONTROL EXPERIMENTS WITH BACILLUS SUBTILIS DURING THE ROOTING PERIOD OF CARNATION CUTTINGS UNDER COMMERCIAL CONDITIONS

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## Abstract

An antagonistic strain of Bacillus subtilis produced in the Research Centre of Biotechnology Berlin (Huber et al. 1987) was tested to control the Fusarium wilt of carnation caused by Fusarium oxysporum f. sp. dianthi. Special attention was given to combined applications of the antagonist with selected fungicides (Bochow et al. 1988) The tests were carried out during the rooting period of carnation cuttings.
Most succesful applications in model experiments were tested under commercial conditions in naturally infested soil. The percentage of well rooted cuttings increased at different levels due to the application of B. subtilis combined with fungicides or $KMnO_4$.

## Introduction

Results obtained in model experiments involving the beneficial use of Bacillus subtilis together with $KMnO_4$ during the rooting period of cold-stored carnation cuttings were investigated under commercial conditions in comparison with a dipping treatment in Captan which has been widely used so far.

## Material and method

1.Carnation cuttings (Scania) were rooted under defined conditions in perlite inoculated with the pathogen in a climate room. After the rooting period the percentages of well rooted cuttings, root weight and disease index were assessed.
2.Carnation cuttings (Scania; n=10400) were rooted in perlite after cold storage under commercial conditions without artificial inoculation of any pathogen. The rooting period was as long as in the earlier model experiments, i.e. 21 days from the usual dipping in Naphtylaceticacid solution. For an assessment of this experiment were obtained the percentages of well rooted cuttings and those of not adequately rooted as well as of cuttings affected by root rot and wilt pathogens.
The applications of B. subtilis($10^7$ cfu/ml) and $KMnO_4$(0,025%) were carried out by pouring on the substrate immediately before planting the cuttings. Treatments with Captan were performed by dipping the cuttings before planting.

## Results/Discussion

Table 1:Results - model experiment

| Treatment | well rooted cuttings(%) | Root weight (relative) | Disease index ( 0-100 ) |
|---|---|---|---|
| Uninoculated control | 100 | 100 | 1,3 |
| Inoculated control | 68 | 70 | 18,9 |
| B. subtilis | 96 | 60 | 13,3 |
| $KMnO_4$ | 93 | 90 | 33,6 |
| B. subtilis + $KMnO_4$ | 100 | 130 | 15,9 |

Table 2: Results - experiment under commercial conditions

| Treatment | Percentages of cuttings | | |
|---|---|---|---|
| | well rooted | not adequately rooted | diseased |
| Captan | 94 | 4 | 2 |
| Captan + B.subtilis | 90 | 8 | 2 |
| B.subtilis | 87 | 8 | 5 |
| KMnO4 | 93 | 5 | 2 |
| B.subtilis+ KMnO4 | 98 | 1 | 1 |

1.In artificially infested soil the treatment with B. subtilis resulted in a decrease of disease severity and in a higher number of rooted cuttings compared to the inoculated control. This effect was promoted by the combined application of B. subtilis with $KMnO_4$ (Obieglo et al. 1990).
2.The conventional dipping treatment of cuttings in captan resulted in an average value of 94% of well rooted cuttings. This result was not improved by the application of B. subtilis in addition to Captan or on its own. This was mainly due to a high number of cold-stored cuttings being affected by root rot pathogens, against which the non-curative antagonist is less effective particularly if the colonization pressure is high, than against the wilt pathogen where it is used bound to a substrate before the infection.

By the application of $KMnO_4$ as a disinfectant, however, nearly the results were obtained as by treatment with captan.
At last it is worth noting the very good result of 98 % of well rooted cuttings obtained under commercial conditions - similarly as in the earlier model experiments - with the combined B.subtilis-$KMnO_4$ treatment of the substrate. Thus the suitability has been confirmed of this combined treatment, in which $KMnO_4$ again acted as a stabilizer for the colonization of the rhizosphere by the antagonist (Bochow et al. 1989).
It is recommended to use B. subtilis in combination with $KMnO_4$ in the rooting period of carnation cuttings instead of dipping these cuttings into suspensions of fungicides (i.e. captan) at concentrations recommended in conventional horticulture.
The application of antagonists and combinations with fungicidal agents in other soilless techniques of carnation culture are being studied (Jacob et al.1988).

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## The use of antagonists to prevent *Phialophora* wilt of carnations in Latvia.

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**Summary.**

In Latvia carnations cover more than 25% of the glasshouse area devoted to flower growing. Sixty percent of the crops are affected by wilt and 45% of the wilt is caused by *Phialophora cinerescens*. Because the use of fungicides to control wilt is not effective, great attention has been given to the search for antagonists against *P.cinerescens*. Microorganisms have been isolated and screened for antagonistic ability against the pathogen and glasshouse experiments have been carried out. It was found that antagonist are most effective if they are applied to the soil before the pathogen is introduced. Several isolates of bacteria including isolates of actinomycetes delayed the appearance of wilt symptoms for several weeks and the severity of symptoms was reduced.

**Introduction.**

Carnation is the most widespread glasshouse crop in Latvia covering about 25% of the glasshouse area. Wilt diseases are causing serious problems especially in industry-type carnations. About 60% of the crops are affected and *P. cinerescens* is the pathogen causing the disease in about 45% of these cases. Much research has been dealing with biological control of *Fusarium* wilt of carnation. Many scientists dealing with this disease have noted the importance of a biological active soil for suppressing the disease (Cebolla, 1984; Garibaldi, 1984). Filippi *et al.* (1984) for example isolated several bacteria from soil which had an antagonistic potential against *F. oxysporum*. Especially a strain of *Bacillus subtilis* (Filippi *et al.* 1987) and fungal antagonists such as *Trichoderma* spp. and non-pathogenic *Fusarium* spp. (Rattink, 1991) have shown promising results in biological control of disease caused by *F. oxysporum*. However, there seem to be no information concerning biological control of *P. cinerescens*. This paper concerns the potential of using bacteria and *Tricoderma* antagonists in the biological control of *P. cinerescens*.

**Materials and methods.**

Microorganisms have been isolated from natural soil and selection of antagonists among the isolates was carried out at the Institute of Biology by Dr. D. Pavlovich. The antagonists have been tested under glasshouse conditions at the Latvian Agricultural Academy. Carnations were inoculated with the pathogen by watering the soil close to the root necks of the plants with conidia suspensions of *P. cinerescens*. The antagonists were incorporated in the soil at different periods - before inoculation with the pathogen, simultaneously, or some days later. The disease was assessed in each treatment 35 weeks after inoculation and the disease development was then followed for the next 5 weeks.

**Table 1.** The effect of different antagonists on wilt disease in carnations caused by *Phialophora cinerescens.* The antagonists were applied before inoculation with the pathogen.
The disease index is scored on a scale from 0= no symptoms to 4= plant dead.

| Treatment with antagonist number | Number of weeks after inoculation | |
|---|---|---|
| | 35 | 40 |
| Control without antagonist | 1.0 | 1.8 |
| Bacteria B131 | 0 | 0.2 |
| Bacteria B177 | 0.1 | 0.2 |
| Bacteria B187 | 0 | 0 |
| Bacteria B246 | 0 | 0.2 |
| Bacteria B270 | 0.3 | 2.0 |
| Actinomycete | 0.2 | 0.4 |
| *Trichoderma* + B246 + B270 | 0.1 | 0.4 |
| *Trichoderma* + B187 | 0 | 0.2 |

The disease intensity was scored according to a scale from 0 to 4, where 0 is plants without any wilt symptoms and 4 is the scoring for totally wilted plants.

**Results and discussion.**

It was found that the antagonist only were effective in controlling the disease when they were applied before inoculation of the plants with the pathogen (table 1). If the antagonists was applied at the same time as inoculation with the pathogen took place or later they did not prevent the disease. Thus, the antagonists should have the possibility to multiply for several days in order to be effective against *P. cinerescent.* Several isolates of bacteria as well as bacteria in combinations with *Trichoderma* spp. have been tested. In the control without antagonists the first wilt symptoms appeared 35 weeks after inoculation and after 40 weeks the typical wilt symptoms could be seen. Plants treated with antagonists showed almost no symptoms after 35 weeks and the plants looked the same even after 40 weeks (table 1). However, all plants were lost by wilt disease later in the season about 50 weeks after inoculation. It can be that the antagonists had difficulty in surviving and remaining active in the substrate for such a long period. Therefore it might be a possibility to apply the antagonists once again or several times in order to control the pathogen throughout the whole season. This approach needs to be studied further.

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# BIOLOGICAL CONTROL OF *ERWINIA* DISEASES OF POTATO.

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## Introduction

*Erwinia carotovora* subsp. *atroseptica* (Eca) is a major pathogen of seed and ware potato crops causing blackleg in the growing crop and soft rot in store. In susceptible varieties the amount of blackleg in the crop is related to the number of bacteria on or in the tubers at planting (Bain *et al*., 1990), but there is no clear relationship between disease in the crop and subsequent colonisation/infection of daughter tubers. This is thought to be influenced by features such as the weather at harvest and the time of lifting. Seed classification (certification) regulations limit the amount of disease in the growing crop in an attempt to minimise disease transmission; there is no effective chemical control for the disease.
Several studies in the USA, The Netherlands and N. Ireland suggest that biological control of *Erwinia* diseases, especially blackleg, using fluorescent pseudomonads may be feasible. The present study was set up to investigate the potential of Scottish bacterial isolates to control blackleg and prevent infection of daughter tubers.

## Materials and methods

Bacterial isolates were obtained from samples of potato tubers, roots and soil from most of the potato-producing areas of Scotland.

### Screening assays

Bacteria were screened for prevention of soft rot (Bain & Perombelon, 1988), osmotolerance (Loper *et al*., 1985) and root colonisation, which involved dipping radish seeds in suspensions of the test bacteria (approx. $10^9$ cfu/ml in 1% xanthan gum) and planting the seeds in test tubes (25 x 145 mm) containing moist sterile vermiculite. After incubation at 27°C for 10 days, the roots were carefully removed from the medium and 10 mm portions plated on to nutrient agar. Root pieces were scored for bacterial colonisation by the presence of bacterial growth on the agar surrounding the root. Fluorescent pigment production was detected by growth on King's Medium B and isolates were checked for pectolytic activity. The leaflet bioassay as described by Chard *et al*. (1990), was also used in some cases.

### Field trials

These were set up at Boghall Farm, Midlothian (1987 and 1988 using the potato variety Maris Bard; 1989, vars. M. Bard & Estima); Kinross, Fife (1988, var. Desiree) and at Gogarbank Farm, Midlothian (1989, vars. M. Bard & Estima). In 1987 and 1988 seed potatoes were vacuum infiltrated with Eca to a level of approximately $10^5$ Eca per tuber; in 1989 naturally infected tubers with a similar level of infection were planted. Trials consisted of five

replicates of four drills (7.5 m) in a randomised block layout. Blackleg symptoms, yield and the soft rot potential of daughter tubers (Colyer & Mount, 1984) were assessed.

## Results and discussion

The potato rot assay was used as the primary screen for antagonism to Eca. Approximately 600 isolates were tested using this method. Organisms were also tested for root colonisation, osmotolerance, fluorescent pigment production and pectolytic activity and these criteria were used for selection for field trial. In 1987 seven isolates representing a range of characteristics were tested in the field. One isolate (J182.7) reduced blackleg in the field. None of the screening methods appeared to correlate with field performance.
Subsequently, a leaflet bioassay was developed as a secondary screen after the soft rot assay. In the 1988 field trials, seven organisms were chosen which differed in the leaflet test. The leaflet bioassay appeared to correlate well with field performance (Table 1); the best isolates (J182.7 and J85.3) from the leaflet test reduced blackleg in field trials.

Table 1. Ranking of selected bacterial isolates in leaflet assays compared to field performance

| Organism | Rank of isolates in leaflet bioassay | | | | Final rank | Field results* |
|---|---|---|---|---|---|---|
| | Eca 1039 | Eca 1039 | Eca 93 | Eca 93 | | |
| J85.3 | 1 | 1 | 1 | 2 | 1 | 3 (4) |
| J182.7 | 2 | 3 | 2 | 3 | 2 | 1 (5) |
| J69.6 | 3 | 4 | 3 | 1 | 3 | 1 (2) |
| J85.4 | 4 | 2 | 4 | 4 | 4 | 0 (2) |
| J69.5 | 5 | 4 | 6 | 5 | 5 | 0 (2) |
| J215.4 | 7 | - | 5 | 6 | 6 | 0 (2) |
| J221.4 | 6 | - | - | - | 6 | 0 (2) |

* Number of field trials where positive control of blackleg was obtained. Numbers in brackets are the total number of field trials for the isolate.

In 1987 and 1988 the yield (weight and numbers of tubers) and soft rot potential of daughter tubers were not affected by any treatment.
Bacteria were applied to tubers immediately prior to planting in 1987 and 1988 in powder formulations (talc-based in 1987 and peat-based in 1988). In 1989, a field trial and glasshouse tests were set up to explore the effect of formulations of the bacteria on antagonism. Peat-based powders (0.5 g per tuber) and granules (1 g per tuber) of isolates J182.7 and J85.3 were tested. Only J85.3 in a peat powder formulation was effective at controlling blackleg in the field (Fig 1) and significantly increased yields of tubers. The granules may not have been applied at a high enough dose rate; glasshouse experiments confirmed the need for at least 2 g

granules per tuber for maximum effect.

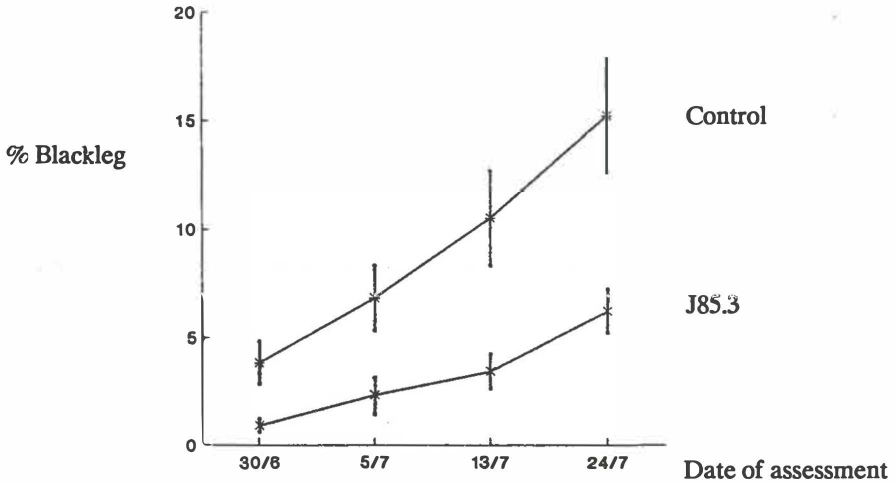


Fig. 1. Incidence of blackleg disease in potatoes treated with a bacterial antagonist (J85.3) compared to the untreated control (1989 trial, Gogarbank).

Conclusions

Bacteria can be used to control blackleg under Scottish growing conditions, but, as in other similar studies, results are variable. In no trial was the soft rot potential of daughter tubers affected by bacterial treatment. The need for a sensitive bioassay for screening isolates cannot be overstated, and formulations of the bacteria can have a great effect on ability of the microorganism to control disease.

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**ANALYSIS OF AGROBACTERIUM POPULATIONS IN CENTRAL AND SOUTHERN ITALY AND SEARCH FOR NATURAL ANTAGONISTS**

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Summary
The great majority of agrobacteria isolated from galled plants in central and southern Italy belong to biovar 2 and are sensitive to agrocin 84. The antagonistic *Agrobacterium* strain K84 was used in biocontrol tests repeated for five years in the same plots and was constantly effective. No selection of virulent resistant population was observed. From 1190 isolates, 22 strains of agrobacteria were selected *in vitro* for antibiotic compound production. None had *in vivo* antagonistic capacities comparable to those of the strain K84.

## Introduction

*Agrobacterium tumefaciens* (Smith and Townsend) Conn induces crown gall on many woody plants and can cause serious losses particularly in nurseries. The disease manifests itself in the form of hyperplasias, which withdraw nutrients from the plant and interfere in the transport of water.
A proven biological control technique against this disease consists of treating the roots (and/or seeds) of the plantlets with a suspension of the strain K84 of *Agrobacterium radiobacter* (Beijerinck and Van Delden) Conn (Kerr, 1980). This strain possesses a high antagonistic capacity, both chemically (production of agrocin 84) and mechanically (competition for infection sites) (Cooksey and Moore, 1982). Today the strain K84 is used successfully in many countries for rose, peach and other stone fruit trees.
However, this method is not effective for cultures like grapevine, chrysanthemum, apple, pear and species of *Rubus*, which are generally attacked by agrobacteria that are insensitive to the strain K84. In addition transconjugant resistant agrobacteria, although rare, may arise (Panagopoulos *et* al.,1979). In our research we have studied the practical application of K84 *in* central and southern Italy, particularly on peach trees. Moreover, we have evaluated the antagonistic capacity of a group of agrobacteria which produce antibiotic compounds.

## Materials and Methods

### 1. Study of agrobacteria populations.

Plants affected by tumors were collected during the last three years from various nurseries in central and southern Italy. Bacteria were isolated from a total of 229 plants, including peach (65%), apple (13,5%) and cherry (8%) as well as some samples of other plants (plum, persimmon, rose, chestnut, daisy). The isolates were identified following Kerr and Panagopoulos' scheme (1977). Virulence of the isolates was tested by inoculation of bacterial suspensions into tomato, datura and sunflower. The following characteristics of each strain were also determined: a) octopine and/or nopaline utilization as the sole carbon and nitrogen source; b) *in vitro* sensitivity to agrocin 84; c) *in vitro* production of antibiotic compounds. *In vivo* sensitivity to K84 of 40 virulent agrobacteria (15 *in vitro* sensitive and 25 resistant isolates) was checked by inoculating into tomato plants suspensions of the virulent strains ($1x10^6$ cfu/ml) mixed with suspension of K84 cells at different ratios.

2. Biological control of peach tree crown gall.
Field trials were repeated for five consecutive years in the same plots and with the same procedural methods, using a virulent biovar 2 agrocin sensitive isolate and K84 cell suspensions. The four different treatments, reported in Tab. 2, were compared. From galled plants, 440 agrobacteria were reisolated and characterized as described above.
3. Study of the antagonistic activity of antibiotic producer strains.
Among 1190 isolates of different origin, twenty-two avirulent strains were detected in vitro for their ability to inhibit growth of biovar 1, 2 and 3 virulent agrobacteria. In vivo tests were carried out with tomato and kalanchoe plants (a virulent to antagonistic strain ratio of 1:1 was used; the K84 strain acted as control).

## Results

1. Study of agrobacteria populations.
A total of 616 isolates were obtained, 81 of which (13.2%) were classified as biovar 1, 482 (78.2%) as biovar 2 and none as biovar 3; 53 isolates could not be allocated in any of the proposed biovars.

Tab. 1 Virulence and sensitivity to agrocin 84 of agrobacteria isolates

| | virulent (335) | | not virulent (281) | |
|---|---|---|---|---|
| | sensitive | insensitive | sensitive | insensitive |
| biovar 1 | 4 | 14 | 14 | 49 |
| biovar 2 | 215 | 88 | 22 | 157 |
| biovar not determined | 13 | 1 | 0 | 39 |
| total | 232 | 103 | 36 | 245 |

Of the isolates, 335 (54.4%), were pathogenic to at least one of the three plant species tested. The majority of virulent bv 2 strains (71%) were sensitive in vitro to agrocin 84, while most of the virulent bv 1 strains (77%) were insensitive. In addition non-virulent isolates were in great part insensitive to agrocin 84 (Tab. 1). The 224 virulent strains tested for opine utilization capacity can be divided into four groups: nopaline utilizers 65.2%, nopaline and octopine utilizers 31%, octopine utilizers 2.5% and non utilizers 1.3%. No correlation between opine metabolism and sensitivity to agrocin 84 was found. In the in vivo tests, sensitive strains showed inhibition already at 1:0.1 ratio of pathogen to K84. The effect increased with the concentration of strain K84 in the inoculum mixture; the reduction of the number of tumors was associated with a decrease in their size. A ratio of 1:1 inhibits only 6 of the 25 in vitro insensitive strains and significantly lowered the number of tumors for 7 isolates. The strain K84 effectively controlled the virulent agrocin insensitive agrobacteria only at the ratio of 1:100; four strains were completely unaffected by the antagonist.
2. Biological control of peach tree crown gall.
The data reported in Tab. 2 demonstrate that the strain K84 had a constant and high efficacy during all five years of the experiment. In this period populations of agrobacteria insensitive to the antagonist were not selected. Therefore, this method is very effective and reliable when applied correctly. The agrobacteria isolated from tumors that developed on treated plants appear to descend mostly from the strain used in the test and which escaped from the control of K84. An equal number of opportunistic non virulent agrobacteria were found in the same tumors. Of 198 virulent bv 2 isolates, 22 (11%), demonstrated insensitivity to agrocin and 3 isolates from 3 tumors were agrocin insensitive and agrocin producers.

Tab. 2 Percentages of diseased plants in the field tests

| | 1985 | 1986 | 1987 | 1988 | 1989 |
|---|---|---|---|---|---|
| T I | 15.7 b | 15.0 b | 35.2 b | 4.0 b | 0.7 b |
| T NI | 16.6 b | 0 c | 2.0 c | 0.8 b | 2.7 b |
| NT I | 46.7 a | 96.4 a | 92.4 a | 53.7 a | 69.7 a |
| NT NI | 2.0 b | 0.6 c | 0.7 c | 0 b | 5.0 b |

TI= treated and inoculated plants; T NI= treated and not inoculated;
NT I= not treated and inoculated; NT NI= not treated and not inoculated.
Values followed by the same letter are not significantly different at 1% level

3. Use of antibiotic producer strains.

The capacity of 22 non virulent strains to produce antibiotics which are active against virulent strains of the three biovars was detected *in vitro*. However, such activity was not found in the *in vivo* trials: all of the antibiotic producer strains failed to inhibit the formation of tumors; only some reduced the tumors' size. The discovery of effective antagonists appears to be a rare occurrence. Nevertheless, from these results, it is necessary to find antagonists which have a range of activity that is different and complementary to that exhibited by the strain K84. This strain has been recently modified genetically in order to block the transfer of the plasmid, that is responsible for the production and resistance to agrocin 84, to other agrobacteria (Jones and Kerr, 1989). However, some countries are not likely to permit the diffusion of genetically manipulated microrganisms in the environment. Also this consideration spurs research for other effective natural antagonists.

## Discussion

- Our data show that biovar 2 is predominant in stone fruit trees in central and southern Italy. In general, these agrobacteria are sensitive to the strain K84, whereas those of biovar 1 are not.
- The strain K84 was constant and effective in the control of peach crown gall in our five-year tests; its repeated use did not select resistant populations.
- Antibiotic producing agrobacteria are found in nature, although not frequently; none of them, however, show *in vivo* the same antagonistic properties as the strain K84.

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# EFFECT OF ANTAGONISTIC *BACILLUS* STRAINS ON *RHIZOCTONIA SOLANI* KÜHN INFECTION OF POTATOES

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**Summary**

Different *Bacillus* strains with highly antagonistic effects against *Rhizoctonia solani* were isolated from soil samples by means of an in vitro screening. The best isolates were examined in greenhouse experiments under modified environment conditions and in field trials to assess their suitability for the biological control of *Rhizoctonia* - disease of potato. The relative occurrence of stem canker of growing potato plants and black scurf on potato tubers was reduced by about 45 % by treatment with *Bacillus spp.* in greenhouse experiments. In field trials the *Rhizoctonia* - disease was diminished by about 76 %. Bacterial antagonists from the genus *Bacillus* are particularly suitable for the biological control of *Rhizoctonia* - disease of potatoes.

## Introduction

*Rhizoctonia* is one of the most serious diseases of potato and other crops. It causes damping off of seedlings, root and stem cankers of growing plants, and black scurf on potato tubers. It is widespread and probably occurs wherever potatoes are grown. Today there are only treatments with different chemical compounds good control measures for *Rhizoctonia* which are both practical and effective (Becker, 1989). Biological control of disease caused by *R.solani* has been reported, and several bacteria and filamentous fungi have been shown to suppress disease and the pathogen (Berggren, 1985). Application of bacterial antagonists from the genera *Bacillus* and *Streptomyces* and mycoparasites from the genera *Gliocladium, Trichoderma* or *Verticillium* on potato effectively reduced disease in laboratory experiments, in greenhouse- or field trials (Beagle-Ristaino and Papavizas, 1985; Davis and Sorrensen, 1986; Jager and Velvis, 1988; Schmiedeknecht, 1990). The present paper will show some possibilities for biological control of the soil- and tuber-borne pathogen *Rhizoctonia solani* KÜHN by bacterial antagonists.

## Materials and methods

Different *Bacillus* strains with antagonistic effects against *Rhizoctonia solani* were isolated from soil samples by means of an in vitro screening. The best isolates (*Bacillus subtilis* IMET 11 434, *B.subtilis* IMET 11 327, *B.subtilis* IMET 11 327 and *Bacillus pumilus* IMET 11 435) were cultivated under submersed conditions in liquid media (glucose-nutrient-broth). The antagonist culture media concentrations were adjusted to $10^7$ ... $10^8$ spores/ml. Then these culture media were used for the biological control of *Rhizoctonia* - disease of potato in greenhouse experiments under modified environment conditions and in field trials. The *Rhizoctonia* - infected

potato tubers were inoculated with culture media. In greenhouse trials the soil was additionally treated with antagonists. The symptoms of *Rhizoctonia* infestation on potato plants were visually evaluated after 8 weeks in greenhouse experiments and after three months in field trials (Schmiedeknecht, 1990; 1991).

## Results

In greenhouse different potato cultivars were treated with bacterial antagonists for protection against soil-borne *Rhizoctonia* - infestation. The occurrence of stem canker of growing potato plants as well as black scurf on potato tubers caused by *R.solani* was on an average decreased by nearly 49 % by treatment with *Bacillus spp.* (Table 1). Also treated plants grew taller and appeared greener than non-treated plants and, at harvest, the fresh weight was higher (48 % stems and leaves; 38 % potato tubers).

Table 1: Effect of treatment of different potato cultivars in greenhouse with *Bacillus spp.* ($10^7$ spores/ml) on reduction of disease severity caused by *R.solani*

| Potato cultivars | Disease index (0...100%) | | Reduction in disease severity (percent) |
|---|---|---|---|
| | Untreated control | *B.subtilis* treatment | |
| ADRETTA | 33,2 | 28,4 | 14,5 |
| FRINGILLA | 50,7 | 17,9 + | 34,7 |
| KARAT | 53,7 | 19,8 + | 63,1 |
| KARPINA | 41,2 | 21,1 + | 48,8 |
| LIBANA | 63,5 | 30,6 + | 51,8 |

+ Calculated 't' value significant at P = 0,05

A number of fungicides used for control of *R.solani* during potato growth were compared with the efficiency of the *Bacillus* strains. The greenhouse experiments showed, that the microbial antagonists from the genus *Bacillus* and the compounds Benomyl and Carbendazim have the same efficiency against *Rhizoctonia*. Only treatment with Pencycuron effectuate better reduction of disease severity than application of biological means (Table 2).

Table 2: The efficiency of *Bacillus spp.* ($10^8$ spores/ml) on reduction of diseases severity caused by *R.solani* in comparison with different fungicides in greenhouse experiments

| Treatment | Disease index (0...100 %) | Reduction in disease severity (percent) |
|---|---|---|
| Untreated control | 38,7 | 0,0 |
| *Bacillus spp.* treatment | 22,8 + | 41,1 |
| Ch. Fundazol [Benomyl] | 25,3 + | 34,6 |
| Funaben [Carbendazim] | 24,7 + | 36,2 |
| Monceren [Pencycuron] | 8,7 + | 77,5 |

+ Calculated 't' value significant at P = 0,05

From 1988 to 1990 in field trials different *Bacillus* strains were used on potato plants for the biological control of tuber-borne *Rhizoctonia* infestation. Seed tubers inoculated with *Bacillus spp.* were dried and planted some days afterwards. In general, the application of antagonistic *Bacillus* strains decreased the incidence of disease caused by *R.solani*. Plants grown from treated seed tubers were only slightly infected at the end of the growing season. Results of the field trials are given in Table 3.

Table 3: Effect of treatment of potatoes in field trials with *Bacillus spp.* ($10^8$ spores/ml) on reduction of disease severity caused by *R.solani*

| Year | Disease index (0...100%) | | Reduction in disease severity (percent) |
|---|---|---|---|
| | Untreated control | *B.subtilis* treatment | |
| 1988 | 45,5 | 12,2 + | 73,2 |
| 1989 | 48,5 | 7,1 + | 84,7 |
| 1990 | 29,9 | 8,8 + | 70,6 |

+ Calculated 't' value significant at P = 0,05

The results of greenhouse and field trials suggest that the severity of stem canker of growing plants and black scurf on potato tubers caused by *R.solani* can be reduced by treatment with efficient strains from the genus *Bacillus*. It is possible therefore that bacterial antagonists might prove very valuable in the future for successful management of plant pathogens.

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# STUDIES ON THE INFLUENCE OF ANTAGONISTIC RHIZOSPHERE BACTERIA ON WINTER WHEAT ATTACKED BY *GAEUMANNOMYCES GRAMINIS* VAR. *TRITICI*

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## INTRODUCTION

Antagonistic bacteria like *Pseudomonas*- species or *Bacillus* strains are intensivly studied for their antagonistic influence on soil-borne plant pathogens and their plant growth promoting effect. For this work the interactions of bacterial antagonists and *Gaeumannomyces graminis* (Sacc.)v.Arx et Olivier var.*tritici* are investigated.This fungus is one of the most important root diseases of cereals worldwide, also in eastern and northern parts of Germany on diluvial soils. Winter wheat, winter barley, and spring barley are attacked and damaged by this pathogen in decreasing range. Yield losses are different and dependend from many facts: Precrop, population density of the fungus, sowing time, climate in autumn and spring, fertilizing management and some more.

In the last years some bacterial strains were selected and tested in several steps in order to use them as biological agents against G. graminis in an arable crop production system with a high share of cereals. Father studies had aim to characterize the different traits ot several strains which determine their antagonistic activity. Some of the results are described.

## MATERIAL and METHODS

Bacterial strains were isolated from *G.graminis*-infested roots from a long term monoculture of wheat without take-all decline. For determination of their antagonistic activity against the pathogen they were tested by seed treatment on wheat in vitro and later in vivo under semisterile conditions in climatic chambers and under nonsterile conditions in a greenhouse. The plants were harvested after 12 weeks and dry matter production compared with infested control plants.

Some favoured strains were tested for three years in trials (site Müncheberg); area of plots was 10 sqm by 8 replications. The influence of antagonists was observed by estimation of the infestation index of plant roots in december, april and june followed by establishing of grain yields.

To investigate the mechanisms of inhibition of G.graminis by bacteria the following methods were used: Production of phytohormons by avenae coleoptile section test (auxins) and barley leaf senescence retardation test (cytokinine like substances); production of siderophores was measured spectrophotometrically in low-iron medium (modified by ALBESA et al.1985) using a Specord (ZEISS) at 400 nm; production of antibiotics was tested in vitro against some soil fungi by agar diffusion technique on CAPEK-DOX medium riched with iron to prevent influence of siderophores.

Table 1: **Production of fluorescent siderophores and antibiotics by bacteria antagonistic against soil-borne plant pathogenic fungi**

| strain | adsorbance (nm) of sid-complex | inhibitionzone (mm) G.graminis | Alternaria sp. | F.solani | R.solani |
|---|---|---|---|---|---|
| **B.subtilis** (living culture) | - | 16,6 | 12,0 | 10,6 | 19,0 |
| **B.subtis** (autoclaved cult.) | - | 12,1 | 8,2 | 6,8 | 13,0 |
| **P.putida** (strain 27) | 1,099 | 0 | 0 | 0 | 0 |
| **P.putida** (strain 17) | 1,684 | 12,0 | 11,6 | 19,7 | 18,6 |
| **Pseudomonas sp.** strain 421 | 0,961 | 0 | 0 | 0 | 0 |
| **Pseudomonas sp.** strain 383 | 1,102 | 2,8 | 3,9 | 13,0 | 0 |
| **Pseudomonas sp.** strain 355 | 1,254 | 0 | 15,0 | 23,0 | 0 |
| **Fungicidin** 750 ppm | - | 20,5 | 22,0 | 13,2 | 17,1 |

Table 2: **Growth promotion of wheat by bacterial strains antagonistic against Gaeumannomyces graminis var.tritici**

| antagonistic strains | production of growth substances: cytokinine/cytokin.-like compounds (%) | IAA (%) | wheat dry matter (%) |
|---|---|---|---|
| **B.subtilis** | 139,8* | - | 149,8** |
| **P.putida** (strain 27) | 155,2* | 116,2 | 145,6** |
| **P.putida** (strain 17) | 145,1* | - | 123,2* |
| **Pseudomonas sp.** (strain 421) | 132,4 | 248,1** | 131,3* |
| **Pseudomonas sp.** (strain 383) | 175,3* | - | 111,3 |
| **Pseudomonas sp.** (strain 355) | 125,8 | 110,7 | 123,3* |

## RESULTS and DISCUSSION

Comparative studies of several bacterial antagonists showed that inhibition of phytopathogenic soil fungi in vitro and growth stimulation of wheat was based on different traits. All tested *Pseudomonas* - strains showed production of fluorescent siderophore-complex with different intensities. Three strains were able to build antifungal compounds which act different from siderophores. The *Bacillus*-strain showed a high antibiotic activity also. The inhibition was efficient against *G.graminis*, but also against *Rhizoctonia solani, Fusarium* sp., and *Alternaria* sp. (tab.1). All tested strains produced phytohormons or phytohormon-like substances in bioassays and stimulated the growth of wheat artificially infested with G.graminis under greenhouse conditions, partly with high significance. But results in vitro from production of siderophores and antibiotics and bioassays showed no correlation to dry matter production in vivo (tab.2).

In outdoor experiments only two strains were efficient in two of three years. After treatment with *Pseudomonas putida* strain 27 yields increased between 8,1 and 19,4 %, and by *Bacillus* between 9,5 and 10,8 % respectivly. Direct application of antagonists on seeds was more efficient than treatment of seeds with peat preparation.

The investigated roots of treated plants showed in december and in april lower number of infested plants and significant decreased number of plants with more than 25 % destroyed root system in this time. Indices of infestation of control plants ranged between 6,3 and 25%, indices of plants treated with *Pseudomonas putida* (strain 27) ranged between 2,7 and 15,6 %. But not in all cases decreased infestation rate corresponded with increased yields.

It seems, that the direct effects of the antagonists, mediated by production of siderophores or / and antibiotics like PCA are connected with indirect interactions like plant growth promotion and stimulation of resistance mechanisms of the plant (BRISBANE & ROVIRA 1988). Early infestation of winter wheat by G.graminis in autumn was prevented by bacterisation of seed but prevention of newly infestation in spring was not possible.

The colonisation of the roots and the successfull expression of antagonistic characters in autumn did not guarantee increasing grain yields in the next year when G.graminis was present.

The manipulation of antagonists as a biological control agent depends on optimal relations in soil, rhizosphere, and climatic conditions. Investigations of these facts and explanation of their influences on antagonism are nessecary for using beneficial microflora.

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**SCREENING SYSTEMS FOR BIO-CONTROL AND GROWTH PROMOTION.**

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## Introduction

Within Zaadunie a microbiology group was established for which the major goals are:
- biological control systems for plant disease, thus providing alternatives for the current chemicals used for plant disease control.
- germination enhancement and growth promotion by seed, young plant or substrate treatments with micro-organisms.

Research is centered around the ECLAIR project 'Biological inoculants for seed/plant establishment', which is devoted to bacterial seed treatments, especially with *Pseudomonas* or *Bacillus* strains. This ECLAIR project is intended as a collaborative effort of three industrial partners (Zaadunie as project coordinator, Limagrain and Irish Sugar) and four publicly funded research groups: Plant Pathology and Molecular Cell Biology (Rijksuniversiteit Utrecht), Food Microbiology (University College Cork) and LEMIR (CNRS, Nancy).

Our first concern is to identify suitable micro-organisms for bio-control and/or growth promotion. Evaluation of the potentiality of micro-organisms, especially bacteria, for suppression of diseases and/or improvement of seed germination and growth is only possible if fast, simple and reliable screening systems are available.

## Results and discussion

### 1. Emergence/growth promotion

Modelcrop is sweet pepper (*Capsicum annuum* cv. Gedeon) as this crop often suffers from problems with emergence and uniformity.
The parameters used for measuring improvement of emergence and/or growth are:
1. germination after 1-2 weeks
2. normal well developed plants after 2-3 weeks
3. fresh weight of the aerial parts after 3-4 weeks.

Results are subjected to analysis of variance (randomized complete block design, 1 factor factorial). After a screening of approximately 200 rhizobacterial strains (mainly from the genera *Pseudomonas* and *Bacillus*) about 7% proved to be stimulating germination and ± 20% was stimulating growth in more than one screening.
Reproducibility is high (60% of the screened strains) although it was difficult to obtain significant differences even with four replications.
Figure 1 is the result of a screening of bacterial strains in this system.
Numbers 1-11 are drenched with approx. $10^8$ cfu of different bacterial solutions per seed, C is disinfected seed.

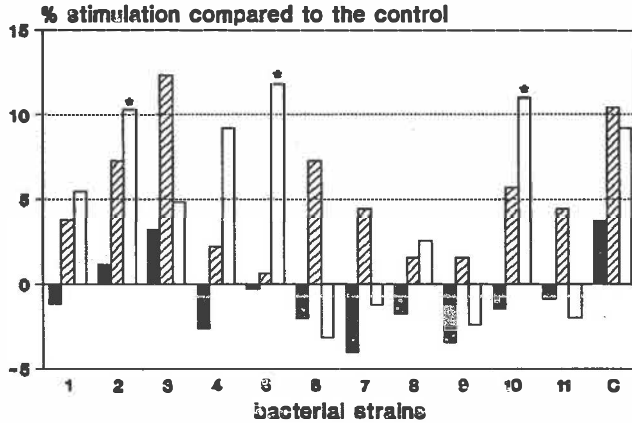


Figure 1: Percentage stimulation (+) or inhibition (-) of emergence/growth after inoculation with different bacterial strains.
(▒) emergence after 14 days, (▨) normal plants after 24 days and (□) mean plant weight after 32 days.

## 2. Biocontrol

### 2.1. Damping-off

Damping-off may be caused by several pathogens, of which the following two, individually or in concert, are the most important: *Pythium ultimum* and *Rhizoctonia solani*. Our modelcrop is radish: *Raphanus sativus* cv. Saxa*Nova.

Development of a reliable bio-assay turned out to be difficult, because in soil artificially infected with *Pythium ultimum*, disease progress was slow and unreliable. Naturally infested soil gave better results (more diseased plants and quicker progress in symptom development) but here the mixture of *Pythium*, *Rhizoctonia*, and probably even others precluded reproducable experiments. Currently, artificial infestation of the soil with a highly virulent isolate of *Pythium ultimum* is attempted.

### 2.2. Fusarium wilt

Model system: *Raphanus sativus* cv. Saxa*Nova and *Fusarium oxysporum* f.sp. *conglutinans*.

The inoculum was prepared according to the method of Löffler and Mouris (1989).This resulted in a stable number of propagules in the soil (± $10^5$/g soil with a 1:100 mixture).

Preliminary tests showed about 50% diseased plants with this soil. Best results were obtained at a temperature of 24°C and sufficient humidity. About 10% of all rhizobacterial strains screened in this system suppressed Fusarium wilt (10-15% more healthy plants).

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# CONDUCIVENESS AND SUPRESSIVENESS OF SOILS

# BIOLOGICAL CONTROL OF *PYTHIUM ULTIMUM* BY INCORPORATION OF ANTAGONISTIC FUNGI IN PEAT SUBSTRATES.

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## Introduction.

Peat substrates, consisting mainly of sphagnum peat moss, are widely used in Danish glasshouses for the production of vegetables and pot plants. Soilborne pathogens including *Pythium* spp. often cause severe disease problems in modern growing systems where soil-less substrates like peat, are used. Possibilities for using chemical control are limited and even with a very high sanitary standard in the glasshouse, damping-off and root rot caused by *Pythium* spp. result in serious losses. Therefore new control measures are being sought, and among these biological control seems promising. The objective of our research is to develop methods for biological control of damping-off and root rot caused by *Pythium* spp. by incorporating antagonistic microorganisms in the peat substrate. This paper will give an overview of some of the approaches and the problems that we have encountered in connection with this work.

## Conducive/suppressive peat.

It has generally been stated that peat substrates are conducive to soilborne diseases because they are not considered to be able to support an actively suppressive biomass (Hoitink and Fahy, 1986). Conducive peat lots were rendered suppressive to several pathogens by amendment with composted bark (Hoitink, 1980). This suppressiveness is believed to be based on the microbial activity and biomass in the amended substrates (Chen *et al.*, 1988). Already in 1982, however, Tahvonen (1982a) had shown that some Finnish light coloured sphagnum peats were highly suppressive against *Pythium* and other soil-borne pathogens. Later he demonstrated that the suppressiveness was due to the presence of *Streptomyces* spp. (Tahvonen, 1982b). Today, isolates of *Streptomyces* from these peat lots are used in the biocontrol agent "Mycostop" produced by the Finnish company Kemira OY (Lahdenperä *et al.*, 1991).
In our experiments more than 30 sphagnum peat lots (light coloured peat from different origins) were tested for their receptivity to *Pythium* disease (Wolffhechel, 1988). Large differences between the peat lots in their receptivity to *Pythium* were found, and some were highly suppressive. Thus it can be concluded that light sphagnum peat can be suppressive. This conclusion is further supported by Hoitink *et al.* (1991).

## Mechanisms of suppression.

In our experiments we found no correlation between the suppressiveness of the peat lots and their pH or their degree of decomposition (Wolffhechel, 1984). A highly suppressive peat lot (Swedish peat, pH 6.3, limed and fertilized) was selected for further study. In order to investigate the mechanism of suppression, this suppressive peat lot was treated in different ways in an attempt to break down the suppressiveness.

Heat treatment at 40°C for 30 minutes had no effect on the suppressiveness, whereas the suppressiveness was broken down gradually by treatments at increasing temperatures above 60°C. Incorporation of benomyl at increasing concentrations into the peat gradually broke down the suppressiveness, but application of various antibiotics active against bacteria had no effect on the suppression (Wolffhechel, 1988).
Benomyl is inhibitory to fungi except most oomycetes and zygomycetes. The sensitivity to both heat and benomyl suggests that the suppressiveness is microbial in its origin and probably caused by fungal antagonists.

**Isolates of microorganisms from the suppressive peat.**

Several microorganisms were isolated from the suppressive peat and reintroduced into peat which had been rendered conducive by heat treatment at 96°C, in order to test their ability to reestablish the suppressiveness. A mixture of bacterial isolates (including *Streptomyces* spp.) applied as an aqueous suspension had no effect on the suppressiveness, whereas fungal isolates belonging to the genera *Aspergillus, Geomyces, Gliocladium Trichoderma*, and *Penicillium* all reestablished suppressiveness (Wolffhechel, 1989). Apparently, the mechanism of suppression in this peat lot was different from the one found by Tahvonen (1982b). It was not tested whether application of mixed populations of bacteria together with the fungal antagonists would add further to the suppression by increasing the general microbial activity in the peat.
There are several examples of successful biological control of soilborne diseases by incorporation of antagonistic fungi in soil (e.g.Sivan *et al.*, 1984) and potting mixes containing peat (e.g. Lumsden and Locke, 1989; Paulitz *et al.*, 1986). Most attention has been given to isolates belonging to the genera *Trichoderma* and *Gliocladium*, and a comprehensive review concerning the potential of these genera for biological control has been published by Papavizas (1985). A formulated product of *Gliocladium virens* has just been registered by the US Environmental Protection Agency (EPA) for use on vegetables and ornamentals in greenhouses against damping-off caused by *Pythium* and *Rhizoctonia* (Lumsden *et al.*, 1991). *Geomyces* spp. have not earlier been described as antagonistic to *Pythium*.

**Production of antagonists.**

Papavizas *et al.* (1984) showed that the method of production of the antagonists and the kind of propagules used (conidia, chlamydospores, mycelium etc.) are important for their efficacy in biological control. Lewis and Papavizas (1984) cultivated the antagonist *Trichoderma viride* on a mixture of bran, sand and water. They showed that actively growing mycelium in close contact with the organic substrate was more capable of proliferating in soil than conidia that were added to soil with bran. However, mycelium does not withstand drying as well as conidia or chlamydospores.
Organisms such as *Trichoderma* or *Gliocladium* can be produced in either liquid or solid media. The amount of biomass and the ratio between conidia and chlamydospores as well as the viability after drying of the product will depend on the method of production and the media used (Lewis and Papavizas, 1983). According to Papavizas *et al.* (1984) and Harman *et al.* (1991), in most cases it will be preferable to produce the antagonists by liquid fermentation compared to solid fermentation, and procedures for producing *Trichoderma* and *Gliocladium* with a high yield of active biomass have been developed (Papavizas *et al.* 1984). In addition to production of the best type of propagules, improvement of the selected organisms by genetic

manipulation should be considered, as discussed by Harman and Tronsmo (elsewhere in this volume).

## Formulation and application of the antagonists.

Different methods have been used to incorporate antagonists in soil. Suspensions of bacteria (Broadbent *et al.*, 1971), fungal spores (Marois *et al.*, 1982), or powdered preparations of fungal mycelium, and sclerotia (Lewis and Papavizas, 1980) have been added directly to the soil. Nowadays, special carrier materials are commonly used. These carrier materials include clay granules soaked in molasses (Backman and Rodrigues-Kabana, 1975), alginate-skim milk beads (Bashan, 1986) and alginate pellets with wheat bran as a food base (Lewis and Papavizas, 1987). Even though compared to organic materials some of these carrier materials are lightweight (Backman and Rodriguez-Kabana, 1975), large quantities are nevertheless needed if the antagonist is to be mixed into the bulk soil. Hence, methods using such materials will mainly be of interest in high-value protected crops such as pot plants and container grown plants.
The formulation of antagonists for incorporation in peat or soil should fullfil certain requirements. It should be economically feasible to produce and easy to handle. The formulation must ensure favourable conditions for the antagonists like optimal pH and good water availability so that the antagonists will become active and have a competitive advantage over the endogenous microflora when they are incorporated in the peat. Enclosement of a food base in the formulation will in most cases favour the antagonist (Papavizas *et al.*, 1984). However, the foodbase must be fully occupied by the antagonist before incorporation in the peat or, alternatively it must be formulated in such a way that it is separated in space from the pathogens in the peat until it becomes fully colonized by the antagonist. When Harman *et al.* (1981) added cellulose as a food base together with *Trichoderma* they found that *Pythium* was stimulated by the food base which resulted in a higher disease attack. Experiments by Kelley (1976) also indicated that when the granules were applied to the soil surface, residual nutrient from the clay granule preparation of the antagonist could be utilized by *Phytophthora cinnamomi* resulting in an increase in disease severity. The strategy employing separation in space might be used in the preparation of alginate beads as discussed by Harman and Lumsden (1990). Another strategy would be to use a food base that can only be utilized by the antagonist; the pathogens being inhibited or unable to utilize it as described by Nelson *et al.* (1988).
Addition of fungicides or antibiotics to which the antagonists are resistant could also be a way of improving the competitive ability of the antagonists as demonstrated by Ahmad and Baker (1987) and Mendez-Castro *et al.* (1983). In this way they were able to improve the root colonization by resistant organisms when the appropriate fungicide was incorporated in the soil. Heat treatment of the peat substrate to suppress the endogenous microflora before incorporation of the antagonists could be another strategy favouring the establishment of the antagonists.

In our work we selected three isolates from the suppressive peat for further study. These were identified as *Trichoderma harzianum* (T3), *Gliocladium virens* (G2) and *Geomyces pannorum*. While we have not yet optimized the formulation of our antagonists for large scale application, we adopted the peat-bran formulation for *Trichoderma* and *Gliocladium* described by Paulitz *et al.* (1986) for experimental purposes in the growth chamber and inoculum of *G. pannorum* was produced by growing the organism in a sand-oatmeal mixture (modified from Widden and

Parkinson, 1978). Both formulations should ensure a favorable pH and a food base fully occupied by the antagonist at the time of incorporation in the peat.

**Tests of the antagonist formulations.**

The peat-bran formulations of *T. harzianum* (T3), *G. virens* (G2) and two *Trichoderma* isolates T12B and T95, (kindly supplied by R. Baker, Colorado State University) were incorporated in conducive peat at the following concentrations: 0, 0.1, 1 and 5% (v/v). Incorporation of 1% repeatedly gave good control of *Pythium* whereas 5% consistently resulted in less control than the 1% treatment.
When the antagonists were applied as conidial suspensions, good control was obtained at $10^6$ and $10^7$ conidia per ml of peat. No reduced control effect was observed at the high concentration even though this concentration is equivalent to the inoculum density in the 5% peat-bran treatment. The two kinds of inoculum might not be directly comparable, though.
The effect of peat-bran alone (without antagonists) or of an autoclaved peat-bran *Trichoderma* formulation was also tested, and both treatments resulted in higher disease incidences (Wolffhechel and Jensen, 1991a).
Whether the reduced disease control at the 5% peat-bran treatment is the result of a direct deleterious effect on the plants by the antagonists or their carrier materials, or whether incorporation of a high amount of peat-bran can create an extra nutrient source for *Pythium* is now being studied. Preliminary results concerning the competition between *T. harzianum* and *Pythium* for organic substrates added to peat is presented by Green and Jensen (elsewhere in this volume).
Good control of *Pythium* disease was obtained after incorporation of sand inoculum of *G. pannorum* to conducive peat at 15 g per litre (Danielsen and Wolffhechel, 1991).

**Water potential in the peat.**

The modern techniques of irrigation used in Danish glasshouse crops, especially in pot plants often result in water contents of the peat temporarily reaching very high levels.
Therefore we tested strains of *G. virens* (G2) and *T. harzianum* (T3, T12B and T95) for their antagonistic activities in peat at different water potentials. The antagonists were applied as 1% peat-bran inoculum to peat with water contents of -6.9kPa, -3.4kPa and -1.5kPa respectively. Best control was achieved at the lowest water content whereas at the higher water contents control was considerably lower (Wolffhechel and Jensen, 1991b). These results may be explained by a lowering of antagonistic activity at the higher water contents. In accordance with our results, Kelley (1976) showed a complete loss of the ability of *T. harzianum* to control *Phytophthora cinnamomi* when it was used in autoclaved soil saturated with water. *Pythium* spp. have a preference for high soil moisture and a tolerance for low oxygen levels (Brown and Kennedy, 1986; Stanghellini and Burr, 1973). However, in our experiment there was also evidence indicating that the plants had been predisposed to *Pythium* attack at the higher water contents. The lowest water content we tested is close to the optimal level recommended for plant growth in peat whereas the two other levels are well above. The experiment illustrates the importance of considering the environmental conditions in the peat if successful biocontrol is to be obtained by incorporation of antagonists in the substrate.

**Plans for the future.**

It is our intention to try to incorporate combinations of different antagonists in peat. One hypothesis is that organisms fitted to the same environmental conditions might have an additive or synergistic effect in controlling disease. On the other hand, a more stable biological control might be obtained under the changing edaphic/climatic conditions in a commercial glasshouse crop by combining antagonists with different environmental requirements. We will also try to incorporate organisms in combinations formulated in a way that will improve both the specific and the general suppressiveness of the peat to *Pythium* spp. In our experiments we will follow the development of the populations of antagonists over time after their incorporation alone or in combinations. The influence of changes in edaphic and climatic factors on the populations as well as on the antagonistic activities will be studied.

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# RELATION BETWEEN SOIL STRUCTURE, *FUSARIUM* LOCATION AND SOIL RECEPTIVITY TO THE FUSARIUM WILT OF FLAX. PRELIMINARG RESULTS.

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## INTRODUCTION

The disease severity of Fusarium wilt varies according to the soil type. Some soils are known as suppressive soils while others are very conducive. Both microbiological and physico-chemical factors are involved in the suppressiveness of a soil. If the former have been clearly demonstrated, through the role of competition for the nutrient among the *Fusarium* populations and between *Fusarium* populations and the whole microflora (Alabouvette, 1989), the latter were only investigated using the soil in its whole and we did not succeeded to get enough informations to explain the role of the physico chemical properties of the soil.
Indeed, there is such a discrepancy in scale between a ten grams soil sample and the microhabitat of the microorganisms than one might have missed the soil property (ies) involved.
For example, the competition issue will depend on the accessibility of the available nutrients for the competing microorganisms (Rosenzweig and Stotzky, 1980), which will in turn depend on the soil structure, resulting from the soil texture and the root progression. Thus, our hypothesis is that the location of the fungi inside the soil aggregates will differ between the soils. These soil aggregates offer a protective micro-environment where the fungi may find more available nutrients resulting from root decomposition than outside these aggregates.
This is the reason why we divided the soil into its aggregates. Then, each class of aggregates (class a : > 200 $\mu$m ; class b : 50-200 $\mu$m ; class c : 20-50 $\mu$m ; class d : 2-20 $\mu$m ; class e : < 2 $\mu$m) were assayed (i) for the number of *Fusarium* and the number of bacteria by plate counts on the appropriate medium, and (ii) the organic C and N availability. Moreover, the whole soil was analysed for its texture, the quantity and kind of clays and its receptivity to Fusarium wilt of flax. The aim of this work is to find, if any, a relation between the soil receptivity and one or more of the parameters tested.

## MATERIALS AND METHODS

Ten soils were chosen within a small range of pH (7 to 8.4.), with various amount of clay and organic matter.
Each soil sample (30 g) was dispersed in sterile distilled water (200 ml) for one hour on a rotary shaker (Bruckert, 1979). Then the suspension was sieved first through a 200 $\mu$m sieve, then through a 50 $\mu$m sieve. That gave class a (> 200 $\mu$m) and class b (50-200 $\mu$m) respectively.
The three other classes c (20-50 $\mu$m), d (2-20 $\mu$m) and e (< 2 $\mu$m) were obtained by sedimentation in water at 4° C either on the bench at normal gravity (c), or in a centrifuge at 100 g for 8 min. (d) or after flocculation with $CaCl_2$ (e).
An aliquote of each class was then used for the microbiological analysis.
C and N analysis are done by the Service Central d'Analyses C.N.R.S. (Lyon - France) and the clay analysis are performed by the "Centre de Pedologie" of Nancy (France). The soil receptivity to fusarium wilt of flax was assessed by adding increasing concentrations

Figure 1

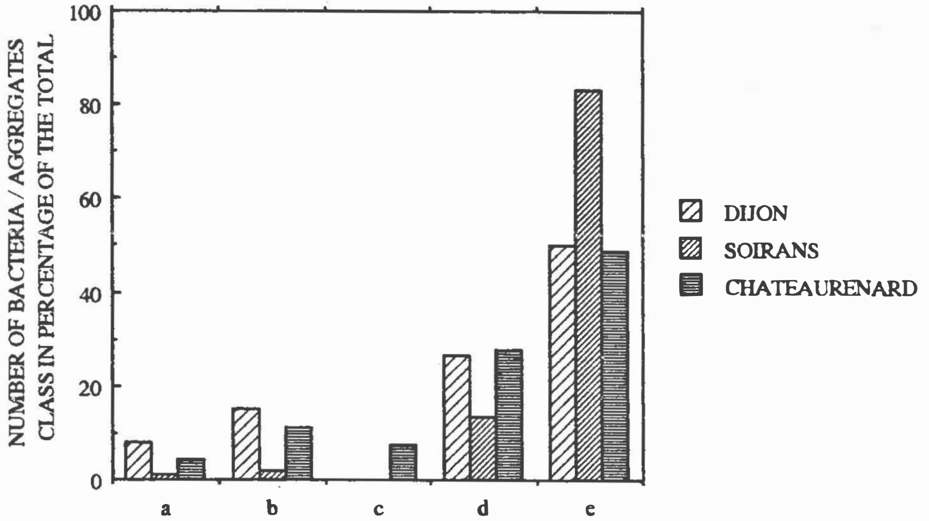
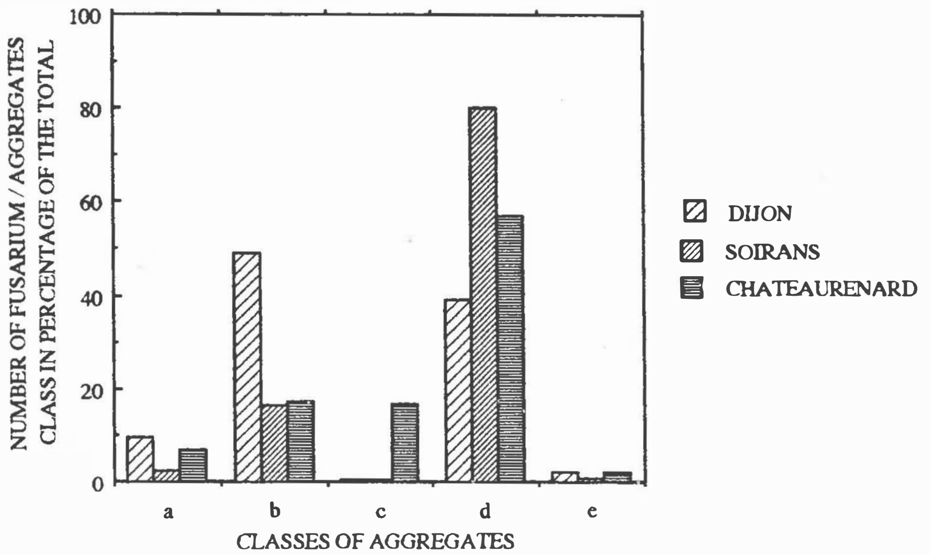



Figure 2



of a pathogenic strain of *Fusarium oxysporum* f. sp. *lini* to the soil which was seeded with flax and by counting the percentage of healthy plants every week.
All the experiments are done at less in triplicate. At this time, we have not got all the results and we will only discuss about the structuration of the aggregates and the distribution of the microorganisms in 3 of the soils.
(i) Dijon and Châteaurenard, two silt clay soils with pH of 8 and 8.4. respectively and organic matter content of 1.7 and 3.9 % respectively.
(ii) Soirans, a loamy soil, with a pH of 7.5 and a high organic matter content of 1.6 %.

## RESULTATS AND DISCUSSION

The texture of each soil was compared with its aggregate distribution. The particles less than 2 µm (e) and at a lesser extent particules ranging from 2 to 20 µm (d) were used for building larger aggregates, specially in class c. This aggregation concerned rather the finest particles in the case of Dijon and Châteaurenard. In the case of Soirans, the build up of aggregates came both from particles from class e and class d. In Dijon a lot of aggregates were produced in the class b.
Soil structuration differed between the soils. It was higher in Dijon because of a higher clay content.
The density of microorganisms per g. of aggregates varied according to the classes and to the soils.
As expected, the highest bacteria density was found in class e, including free bacteria and bacteria binded to clay minerals, while, surprisingly, the bacteria density was the lowest in class c, even if it was still high (about $10^6$ b/g aggregates) for Châteaurenard.
As well, the *Fusarium* were very few in class c, specially in Soirans and Châteaurenard, while the *Fusarium* populations were more dense in class d.
So, it appears clearly that the distribution of the microorganisms is not uniform through the soil (figure 1 and 2).
We are not yet able to conclude on the physico-chemical basis of suppressiveness. We are expecting a lot from the C and N analysis of the aggregates to explain such a distribution of the microorganisms, the C/N ratio being possibly related to the clay nature and pH (Rosenzweig and Stotzky, 1979). We hope that the explanation of the differential distribution will lead us to the explanation of the differences in soil suppressiveness.

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**DEVELOPMENT OF A BIO-ASSAY TO ESTIMATE SOIL-RECEPTIVITY TO *RHIZOCTONIA SOLANI* IN TULIP AND IRIS.**

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## Summary

In the flowerbulbs tulip and iris the 'Rhizoctonia Disease' can be caused by two anastomosis groups of *Rhizoctonia solani*, which differ in their ecological abilities and needs. To reduce the input of pesticides, a durable integrated control management is needed. Therefore, a research project is started to detect and analyse differences in receptivity to 'Rhizoctonia Disease' between flowerbulb fields. For consistent results an accurate bio-assay is necessary. The development of the testing device is described.

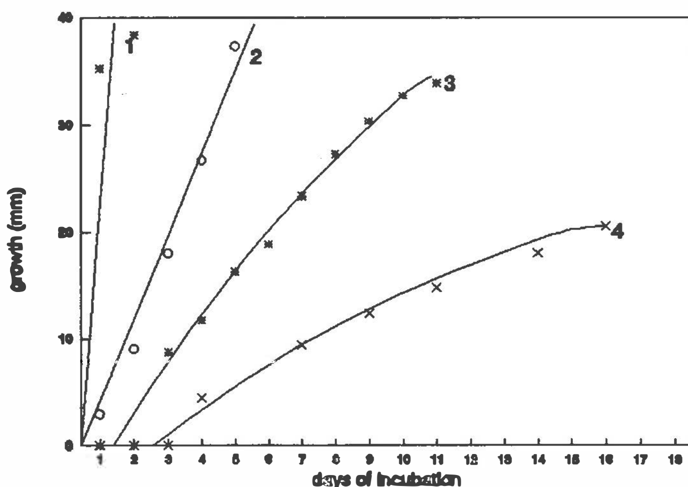
## Introduction

During the past decade, the problem 'Rhizoctonia Disease' in the flowerbulbs tulip and iris has increased. The disease can be caused by two anastomosis groups (AG's) of *Rhizoctonia solani*, which differ in their ecological abilities and needs. For environmental reasons, the use of chemical control agents has to be reduced and authorization of some fungicides has been terminated. Frequent use of a few remaining fungicides will quickly lead to fungicide resistance. Flowerbulb cultivars with resistance to *R. solani* are not known. Hence, a more durable biological/integrated control strategy is necessary.
It is well documented that the incidence of soil-borne fungal diseases may vary considerably between different (agricultural) fields. This variation originates from differences in abiotic as well as biotic soil components which partly may be induced by differences in cropping history. The present project intends to detect and analyse the causal mechanisms behind differences between field soils in receptivity to 'Rhizoctonia Disease' in order to develop a system to induce disease resistance. Thus, the development of a bio-assay is essential for:

- ecological characterization of the pathogen(s),
- estimation of the receptivity of field soils,
- analysis of the mechanisms involved.

## Characterization of the pathogens

For a successful biological control the precise ecological characterization of the pathogens is essential. 'Rhizoctonia Disease' in tulip and iris can be caused by two AG's of *R. solani*. Previous greenhouse experiments at the Bulb Research Centre in Lisse have revealed that pathogenicity of *R. solani* AG's may depend on temperature. In greenhouse studies 'warmth preferring' isolates of *R. solani* caused disease at temperatures exceeding 13°C, whereas 'cold preferring' isolates of tulip were pathogenic mainly at soil temperatures below 13°C (Doornik, 1981). Later, Loerakker and van Dreven (1985) concluded 'warmth preferring' isolates to belong to *R. solani* AG-4. This is a polyphagous plantpathogen with a worldwide distribution and especially attacks hosts in the warmer regions. Loerakker and van Dreven also suggested 'cold preferring' isolates from tulip to be classified as a (new) subgroup of *R. solani*, AG-2id (AG-2id: AG-2 in determination). Thus, in fieldgrown tulip and iris 'Rhizoctonia Disease' may be caused by *R. solani* AG-2id and AG-4, which differ in host range and ecological needs and abilities.
Pilot experiments confirmed that the average growth rate of isolates of AG-2id and AG-4 differs at different temperatures on malt peptone agar (fig 1).
In order to come to a successful control method, a thorough ecological characterization of the pathogens involved is necessary. Therefore, a testing device which allows bio-assays under controlled soil humidity and soil temperature is being developed.




1)AG-4, T=20°C; 2)AG-2id, T=20°C; 3)AG-2id, T=9°C; 4)AG-4,T=9°C.

Fig 1. Average growth of *R. solani* AG-2id (AG-2id: a subgroup of AG-2 in determination, isolated from tulip), and AG-4 on malt peptone agar at two temperatures (preliminary results). Average growth: AG-2id: 5 isolates, 4 replicates; AG-4: 4 isolates, 4 replicates.

Soil-receptivity

The site-specific expression of *R. solani* in the field is a dynamic process and depends on local combinations of soil factors. These combinations probably determine the soil-receptivity of a certain site. "The concept of *receptivity of soil to a disease* comprises all those soil factors which modify the activity of a pathogen" (Alabouvette et al., 1981). A first step in this research is to verify whether differences in soil-receptivity between flowerbulb fields are existing and usable phenomena. The development of a bio-assay to estimate the receptivity of soil samples therefore is essential.

Bio-assay

For *R. solani* in tulip, disease-expression is greatly affected by soil temperature and probably to a lesser extent, by soil humidity. These factors should be adjustable and constant during the bio-assay. Therefore, a system is being developed which regulates the water potential by computer and the soil temperature by a pump-cooling unit during the tests (fig 2).
The system consists of a double-walled stainless steel container. The temperature of the soil can be controlled by a pump-cooling unit, which circulates a fluid inside the double wall of the container. Soil samples can be placed on adjustable bottom plates or on oasis inside the container. The soil water potential is constantly monitored by mini-tensiometers connected to a computer. When the water potential exceeds a setpoint, the computer opens the valves. Water is supplied immediately since the waterdosage system is under constant pressure. Water can be supplied along the bottom (with oasis as buffer), on top through mini-nozzles, or in combination.
Previous experiments carried out by Oyarzun & Dijst (1991) compared the computerised testing system with a conventional bio-assay. The results of the testing device (using six 160 ml mini-containers per container) and the conventional test (using 2.6 l pots) correlated well.
At the moment the system is being adjusted to estimate the receptivity of soil samples to *R. solani*.

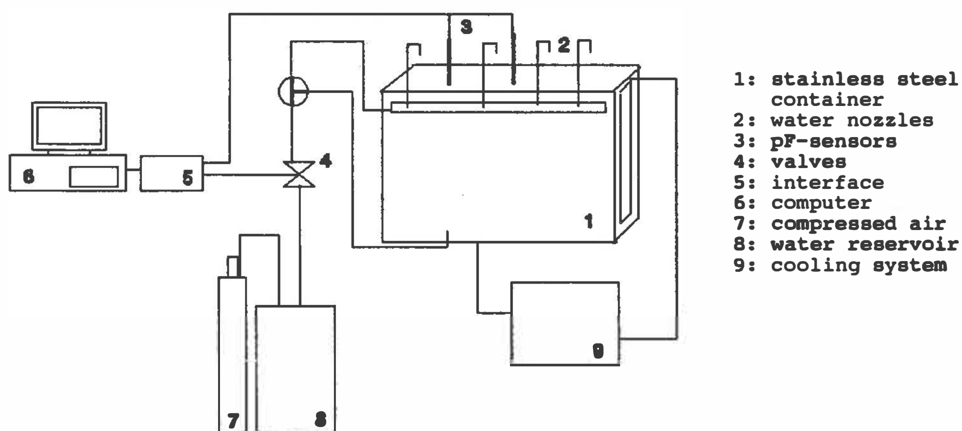



Fig 2. Diagram of a computerised testing device. The soil water potential is computer controlled. The soil temperature can be adjusted independently of the environment.

## Expectations

The ultimate aim of the project on *R. solani* in the flowerbulbs tulip and iris is to develop an integrated/biological control strategy, using differences in soil-receptivity. Analysis of the causal mechanisms is expected to yield the key towards a durable control strategy. For reproducible results the development of a bio-assay with controlled soil humidity and soil temperature is necessary. By means of this testing system we expect to:

- elucidate ecological abilities and needs, which determine disease-expression by the pathogenic AG's involved,
- classify flowerbulb soils according to their receptivity to *R. solani*,
- detect and analyse the causal mechanisms of differences in receptivity between soils,
- predict the efficacy of a potential biological or cultural control method.

At the long term these steps have to lead to a biological/integrated control strategy, which induces suppressiveness of a soil to the disease.

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*RHODOCOCCUS FASCIANS:* INVESTIGATIONS ON ITS DECLINE DURING CONTINUOUS CROPPING OF LILY AND THE MICROBIAL ANTAGONISM TOWARDS IT

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## Introduction

*Rhodococcus fascians* (Tilford) Goodfellow 1984 (syn. *Corynebacterium fascians*) is a plant pathogenic bacterium with many host plants. It causes fasciation on sweat pea, and leafy gall on many other plants. Buds or short, thick, distorted stems with misshapen leaves are produced, often at the soil line. The geographical distribution of the disease is mainly in the Northern hemisphere. The pathogen interacts with the plant hormone balance of the host. In The Netherlands, the pathogen caused some outbreaks in lily and dahlia crops in the late seventies and early eighties and it is now, again, a pathogen of minor importance. The severe outbreaks of the disease in lily led to the design of field experiments in order to study the effect of crop rotation on the development of the disease.
These field experiments gave a good opportunity to investigate the ecology of the pathogen and the microbial antagonism towards it. The aim of this study was to study the effect of the rotation cycle on the pathogen population in the soil, and to obtain information on the antagonism against *R. fascians* and the application of antagonists in a biocontrol experiment. The aspects of this study were: 1. to assess the *R. fascians* population density in the rotation experiment; 2. to study the existence of microbial antagonism against *R. fascians*; 3. to isolate antagonists from the soil; 4. to quantify the antagonist population in soils from diseased and healthy stands of lily, and 5. to apply antagonists in a biocontrol experiment.

## Materials & Methods

- *Isolation of R. fascians from field soil*. The pathogen was isolated from the soil after incubation a 1:10 suspension in sterile distilled water at 4°C. After incubation, aliquots of the suspension were plated onto D2 medium (Kado & Heskett, 1970) with additional 100 ppm potassium dichromat.
- *Demonstration of the antagonism towards R. fascians*. The existence of antagonism against *R. fascians* was demonstrated by the growth of the bacterium on a cellophane film covering a mixture of soil and water agar (see Fig. 1). A drop of *R. fascians* in saline or nutrient broth ($10^8$ cells $ml^{-1}$) was spread onto the membrane. Incubation 8 days at 27°C.
- *Isolation of antagonists against R. fascians*. Antagonists were isolated by a method based on the technique of Sillman & Casida (1986) used for the isolation of bacterial predators. A millipore filter (1.2 $\mu m$) was placed onto soil; a suspension of *R. fascians* has been infested onto the filter; incubation 5 to 6 days at 26°C; the growth on the filter was diluted in saline and plated onto plates with a 10% strength yeast peptone glucose agar (YPGA) with $5.10^7$ *R. fascians* cells $ml^{-1}$ .
- *Quantification of the antagonist population*. The number of antagonists was estimated by the Most Probable Number method (McGrady, 1918). Eight samples of two soils with healthy and diseased lily stands respectively were used. From each sample a dilution series was made. Aliquots of the dilution steps (10 $\mu l$) were plated on YPGA containing *R. fascians*. Halo formation indicated the presence of antagonists in the dilution.

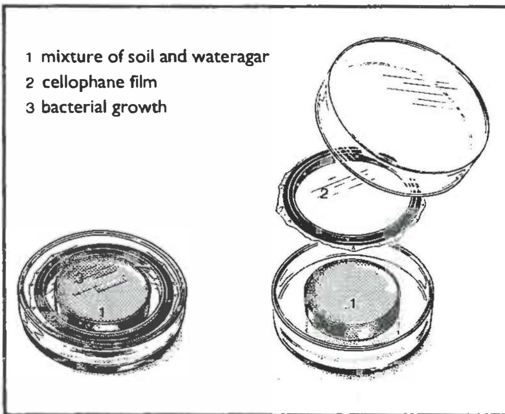


***Figure 1. Demonstration of antagonism against R. fascians***

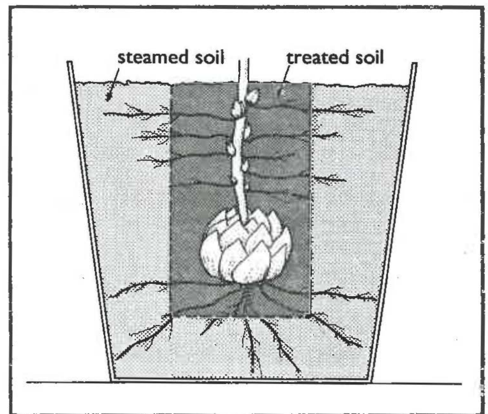


***Figure 2 Infestation of the soil in the biocontrol experiment***

- *Biocontrol experiment*. Six bacterial antagonists were used in a biocontrol experiment with the lily cultivar Conneticut King as host plant. A five-litre container was filled with steamed, recolonized potting soil with on top of the mother bulb a column (9 cm in diameter and 16 cm high) of treated ($10^7$ cfu $g^{-1}$) or non-treated soil. Treatments with healthy or diseased mother bulbs in combination with the pathogen and/or antagonists were studied. After incubation during 80 days at 20-24°C in a greenhouse, the daughter bulbs (bulbils) were harvested and the number of diseased bulbils was counted, and the percentage diseased bulbils calculated. The number of bulbils per treatment varied between 46 and 101.

## Results

- *Isolation of R. fascians from soil*. *R. fascians* was most dominant in the 1:3 rotation (one year lily followed by three years fallow), and to a lesser extent in the soil that had been continuously cropped with lily with $2*10^6$ and $1*10^5$ cells $g^{-1}$ soil, respectively. In the 1:1 rotation intermediate numbers were found ($4*10^5$ cells $g^{-1}$ soil).
- *Demonstration of the antagonism towards R. fascians*. Since it is rather difficult to measure the antagonistic activity from the soil microflora against *R. fascians*, an impression of the growth of *R. fascians* is given in Fig. 3. The extra nutrients stimulated the growth of the pathogen on the cellophane film significantly.
- *Isolation of antagonists against R. fascians*. It was relatively easy to isolate bacterial antagonists of *R.*

sterile soil
non sterile soil
*R. fascians*
*R. fascians* + extra nutrients

*Figure 3 Growth of R.fascians on cellophane film as influenced by antagonistic activity*

*fascians*. Sixteen bacterial antagonists were isolated, and further tested for their antagonistic activity on nutrient plates with the pathogen. Six different isolates were chosen to use in the biocontrol experiment. The choice was based on the habitat of the colonies and the size of the formed halo's. The bacterial antagonists were not identified.

- *Quantification of the antagonist population*. The numbers of antagonists in soil with a healthy crop of lily were significantly higher (Wilcoxon test, $P<0.05$) than in the soil with a diseased lily crop. The density of antagonists was $3.36*10^5$ and $3.9*10^4$ halo forming units per gram soil, respectively.
- *Biocontrol experiment*.

The results of the biocontrol experiment are given in table 1.

Table 1. Effect of treatments with antagonists on the infection of bulbils of lily.

| | Treatment | Percentage diseased bulbils | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Antagonists | | | | | |
| | | | C1 | L2 | N1 | R1 | Z1 | A |
| 1 | d.m.b. in s.s. [1] | 22 | - | - | - | - | - | - |
| 2 | d.m.b. in s.s. + *R. fascians* | 21 | - | - | - | - | - | - |
| 3 | d.m.b. in s.s. + antagonist | - | 30 | 17 | - | - | - | - |
| 4 | d.m.b. in s.s. + *R. fascians* + antagonist | - | 22 | 31 | - | - | - | - |
| 5 | h.m.b. in d.s. | 44 | - | - | - | - | - | - |
| 6 | h.m.b. in d.s. + antagonist | - | 33 | 48 | - | - | - | - |
| 7 | h.m.b. in s.s. | 29 | - | - | - | - | - | - |
| 8 | h.m.b. in s.s. + *R. fascians* | 21 | - | - | - | - | - | - |
| 9 | h.m.b. in s.s. + *R. fascians* + antagonist | - | 38 | 30 | 42 | 43 | 22 | 23 |
| 10 | h.m.b. in s.s. + antagonist | - | 26 | 21 | 26 | 32 | 16 | 13 |

[1]) d.m.b. = diseased mother bulb; h.m.b. = healthy mother bulb; s.s. = steamed soil; d.s. = diseased soil; - = not tested.

The results show a large variation in the percentage of diseased bulbils. In the first four treatments with diseased mother bulbs in steamed soil the inoculation of the soil with the pathogen did not result in more diseased bulbils although a virulent strain was used. Addition of antagonist C1 to the soil gave more diseased bulbils. Simultaneous inoculation of antagonist C1 and pathogen gave a lower percentage of diseased bulbils. The results with antagonist L2 gave inverse results.

Diseased soil (treatments 5 and 6) seemed to be more efficient in inducing diseased bulbils. Antagonist C1 had some reducing effect on the number of diseased bulbils whereas antagonist L2 had not.

Healthy mother bulbs in steamed soil gave also diseased bulbils. This indicates that "healthy" stands for healthy looking. Inoculation of the soil with the pathogen and antagonists simultaneously seemed to induce more diseased bulbils as compared to inoculation with antagonists only (treatments 9 and 10).

## Discussion

One of the most obvious results of this study is the indication that continuous culture of lily results in a lower density of the *R. fascians* population in the soil. Whether the pathogen induces the development of an antagonist population is not known. Inoculation experiments in which *R. fascians* was added to soil in order to induce antagonism did not result in an increase of the population of antagonists. Inoculation of the soil with antagonists gave no significant reduction of the disease. Similar results were obtained by Jacobs and Mohanty (1951) with sweet pea.
The large variation in the results of the biocontrol experiment asks for another approach in future experiments. Firstly, the induction of diseased bulbils needs to be improved. Either by inoculation healthy mother bulbs with the pathogen or by the use of diseased mother bulbs. The results have to show a minimum of variation. Secondly, it is necessary to know that healthy mother bulbs are not latently infected. Thirdly, an experimental design should be used that takes into account the large variation.
The choice of antagonists remains a critical point. This pilot experiment also indicates that antagonistic activity *in vitro* will not always result in visible antagonistic activity in *in vivo* situations.


## Acknowledgement

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# TAKE-ALL DISEASE OF WINTER WHEAT - FIELD EXPERIENCE IN THE USE OF BIOLOGICAL CONTROL

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## Introduction

It has been suggested (Deacon, 1988) that it is unrealistic to expect an introduced biological control agent (BCA) to withstand the high level of take-all (*Gaeumannomyces graminis* var. *tritici*) inoculum that might be present in a second to fourth successive wheat crop after a non-cereal "break" crop. In the work described here, bacterial and fungal BCAs were introduced in two successive winter wheat crops, starting with the first wheat following a "break" - with the aim of preventing an increase in *Ggt* inoculum. One of the experiments also included a treatment to investigate whether the antibiotic avenacin, produced by oat (*Avena sativa*) roots (Holden, 1980), could confer protection against take-all to surrounding wheat plants when grown in mixture.

## Experiment one

### 1. Method

A field trial on silty clay loam, pH 7.1, tested the activity of the fungal BCA *Microdochium bolleyi*, applied as a seed treatment (by Deacon, Edinburgh University), and the effect of growing oats in mixture with the wheat, against take-all. The treatments for this two-year trial (harvest years 1988 and 1989) are shown in Table 1. A non-cereal crop was grown on the site in the year preceding the start of the trial.

Table 1. Treatments

| Treatment number | Treatment in: Year 1 (low take-all risk) | Year 2 (high take-all risk) |
|---|---|---|
| 1. | Untreated seed | Untreated seed |
| 2. | Organomercury treated seed | Organomercury treated seed |
| 3. | Organomercury treated seed | *M. bolleyi* treated seed |
| 4. | *M. bolleyi* treated seed | Organomercury treated seed |
| 5. | *M. bolleyi* treated seed | *M. bolleyi* treated seed |
| 6. | Wheat + oat seed mixture* | Wheat + oat seed mixture* |

* - 10% oat seed mixed with 90% wheat seed by weight.

The oats were removed at the end of April in both years by an application of difenzoquat. Complete kill was not achieved and hand roguing was required before harvest. The trial was of randomised block design with six replicates. Plots were 5 m by 12 m. Take-all assessments were carried out on washed roots of 20 randomly selected plants from each plot in February and May.

2. Results

In the first year of the trial, ten plants per plot were sampled in March to assess establishment of M. bolleyi. The fungus was consistently recovered from the roots of plants from treated plots and not from untreated plots. A negligible number of roots were affected by take-all in the first year of the trial. In the second year, substantial take-all infection developed (Table 2).

Table 2. Effect of M. bolleyi seed treatment and oat mixture on take-all and grain yield

| Treatment number | % seminal roots affected | | % crown roots affected | Yield (t/ha @ 85% DM) |
|---|---|---|---|---|
| | 7 February | 8 May | 8 May | |
| 1. | 15.2 | 50.9 | 7.1 | 4.83 |
| 2. | 10.5 | 42.9 | 8.9 | 5.84 |
| 3. | 8.9 | 37.7 | 6.6 | 5.81 |
| 4. | 7.3 | 50.7 | 6.8 | 5.60 |
| 5. | 8.1 | 42.3 | 9.5 | 5.97 |
| 6. | 8.6 | 49.5 | 7.5 | 4.63 |
| SED (25 df) | 2.15 | N.S. | N.S. | 0.445 |
| LSD (5%) | 4.4 | | | 0.92 |

N.S - not significant at the 5% confidence level.

Experiment two

1. Method

Field trials at four sites tested the effect of a fungal, and two bacterial, BCAs against take-all on winter wheat. Soil types were peaty loam, loamy sand, silty loam and sandy clay loam of pH 6.5, 7.4, 8.3 and 7.9 respectively. The trials were of a randomised block design with six replicates. Plots were a minimum of 6 m by 12 m. M. bolleyi was applied as a seed treatment by Deacon. The two bacterial BCAs, Pseudomonas fluorescens NRRL B-15132 (2-79) and NRRL B-15134 (13-79) were supplied by Weller, Washington State University and were converted into a peat-based formulation containing $10^9$ cfu $g^{-1}$ peat, by the Agricultural Genetics Company, Cambridge. This formulation was applied to the seed immediately prior to sowing. Testing of residual seed showed a count of approximately $10^4$ cfu per seed. The trials each consisted of treatments where the BCAs were introduced only in the second year of the trial, and other treatments where the BCAs were introduced in both the first and second years (harvest years 1989 and 1990). Non-cereal "break" crops were grown on all four trial sites in the season preceding the start of the experiment. Take-all assessments were carried out on washed roots of 30 randomly selected plants from each plot in autumn/winter, spring and summer.

2. Results

The proportion of roots affected by take-all varied widely between sites and years, ranging from 0.5% to 49% of crown roots affected (in untreated plots). Despite this wide range of infection pressure and the wide range of soil types, none of the BCA treatments significantly ($P<0.05$) suppressed take-all at any site in either of the two years. Untreated grain yields ranged from 9.45 t $ha^{-1}$ to 3.77 t $ha^{-1}$ (the latter on the loamy sand site in 1990). None of the BCA treatments had any significant ($P<0.05$) beneficial effect on yield in either of the two years of the experiment.

## Discussion

In experiment one, M. bolleyi seed treatment significantly reduced early season take-all, even where the treatment had only been applied the previous season. Admixture with oats also significantly decreased the disease. However, these treatment effects did not persist to mid-season, and similar effects were seen with organomercury seed treatment - a chemical not normally noted for take-all control. Both BCA and organomercury treatments appeared to improve seedling establishment, possibly due to an effect on Fusarium seedling blight (Spiegel & Schonbeck, 1991) and this may account for part of the yield responses seen. Oat admixture reduced yield, presumably due to a plant competition effect. Possible reasons for the poor persistence or absence of control seen in these trials include: i) High soil pH increasing the dose of phenazine-1-carboxylic acid required to inhibit Ggt (Brisbane & Rovira, 1988), ii) The use of peat in the bacterial BCA formulations, reducing their efficacy (Huber et al, 1989), and iii) Using BCAs not specifically selected for adaptation to the soil conditions encountered. Where 2-79 and 13-79 have been used in a soil of low microbial, chemical and physical complexity, good persistence of take-all control has been achieved (Baldwin et al 1991).

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# DETERMINING THE RISK OF DAMPING-OFF IN SUGAR BEETS.

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## Introduction

Damping-off was the first recognized disease in sugar beets, described as "pied noir" by de Dombâsle (1821) who believed that the disease was caused by spring frost and the quality of the soil. Today it is wellknown that damping-off is caused by fungi, some of which seedborne, e g Phoma betae, some soilborne, e g Aphanomyces cochlioides, Rhizoctonia solani and Pythium spp.
The fungi attack the sugar beet before as well as after emergence, the former killing the seedling and the latter either killing or seriously damaging the seedling, depending on how early in development the attacks begin. The post-emergence symptoms are blackening hypocotyls losing their turgor, sometimes as in the case of A. cochlioides, causing the bases of the cotyledons to blacken as well. After having four to six true leaves the plant usually is on the safe side although attacks at a later stage can cause temporary set-backs.

In 1985 the problem was brought up to date when a large number of Swedish sugar beet fields were devastated by the disease. A joint project was initiated by the cooperation committee of the Swedish sugar beet growers and the department of plant and forest protection. The aim was to determine the distribution of the causing fungi and to investigate the possibilities of forecasting attacks. Similar investigations have been made in other countries (Schäufele & Winner, 1979 and Vestberg, 1984).

## Methods

The investigations have mainly been performed as tests of soil samples. All samples were sown with 50 untreated seeds of the variety Hilma, divided into four pots per sample. The pots were placed in a climate chamber with the following climatic conditions: Temperature, at night 17°C, at day 23°C; Relative air humidity 70-80 % ; Day length 16 hours; Light 20 000 lux. Water was given three times a week. The pots stayed in the chamber for four weeks. Diseased seedlings were removed according as symptoms arose.

To be able to compare different soil samples a damping-off index was designed. The index is time based, i e it considers and places the main focus on rapid heavy attacks whereas attacks at a later stage (predominantly non-lethal) are given less importance. The index is thus built on the amount of attacked seedlings (%) at days 7, 14, 21 and 28 (as7, as14, as21 and as28 respectively).

$$\text{Index (0-100)} = \frac{3*(as7) + 3*(as14-as7) + (as21-as14) + 0.5*(as28-as21)}{3}$$

## Results and discussion

In soil samples from 50 farms, Aphanomyces cochlioides, Pythium spp. and Rhizoctonia solani were the dominating pathogens causing damping-off. Phoma betae and Fusarium sp. were found to a minor extent. Due to the climate installed the test seemed to favour attacks of A. cochlioides.

Comparing different fields of the same farm clearly pointed out the danger of having a strained crop rotation (table 1). The table indicates that the more years between beet crops the lesser is the risk of damping-off.

**Table 1. Damping-off frequency (%) and index for years since last beet crop. Level of significance 55% and 75% respectively. Fields of ten farms.**

| Years since beet crop | Frequency % | Rel no. | Index 0-100 | Rel no. | No. of fields |
|---|---|---|---|---|---|
| 1 | 82.4 | 100 | 53.6 | 100 | 8 |
| 2 | 80.8 | 98 | 60.2 | 112 | 7 |
| 3 | 70.5 | 86 | 48.3 | 90 | 10 |
| 4 | 76.1 | 92 | 44.4 | 83 | 12 |
| 5 | 45.4 | 55 | 24.8 | 46 | 9 |
| 6 | 46.4 | 56 | 28.4 | 53 | 2 |

All of the ten farms in the trial contained at least one field that had suffered from attacks. These fields were unevenly distributed into all six year categories. In order to get a somewhat better correspondence the fields were divided into three groups according to table 2. In this way the long term effects of the specific year of damping-off (different at different farms) is lost. As shown in table 2 the documented damping-off fields are significantly higher in index than the others are. Fields with sugar beets as previous crop form a group of their own, because of their probable propagation of pathogens influencing the results.

**Table 2. Damping-off frequency and index for three groups of fields from ten farms. Level of significance 97.9% and 99.2% respectively.**

| Fields | Freq. % | Rel no. | Index 0-100 | Rel no. | No. of fields | Duncan |
|---|---|---|---|---|---|---|
| A Known damping-off | 88.0 | 100 | 65.5 | 100 | 12 | * A>C |
| B Beet crops previous year | 82.7 | 94 | 53.6 | 82 | 8 | |
| C All others | 59.2* | 67 | 33.7* | 51 | 28 | |

The results suggest that fields once attacked are highly prone to suffer from repeated attacks.
Can these results be used to decide whether growing sugar beets or not at a specific field is advisable? - Since the disease is very much depending on the outer conditions, i e temperature and moisture, it is almost impossible to decide if there will be attacks in the specific field. However, you could decide if there is a **potential danger** of attacks. An evaluation of the risk of damping-off is shown in table 3.

**Table 3. Evaluation of the risk of damping-off. Index 0-100.**

| Index | Evaluation of risk | Comments |
|---|---|---|
| 0- 20 | No risk | - |
| 20- 40 | Small | Normally no problems |
| 40- 70 | Medium | Growing sugar beets could be hazardous |
| 70-100 | Large | If the conditions are favourable, damping-off is highly likely. |

Previous investigations were performed after soil sampling in the spring. To be able to advise the farmer before sowing, it is essential to have the result much earlier. Investigations were therefore made to compare sampling in winter and spring respectively. All in all fifteen fields were sampled on two occasions (December - January and March - April). Analyses of regression show that the coherence is fairly good, the determination coefficient being $r^2$=0.853. Only in one case would the advice according to table 3 be altered. If this case is removed, $r^2$=0.902.
Five out of fifteen tested samples indicated large risk of damping-off. Neither of these five had any problems in the actual sugar beet fiels, however. The reason for this was that the climatic conditions were not favouring attacks. The spring was very warm but also very dry. A. cochlioides requires temperatures above 21°C (McKeen, 1949) as well as high soil moisture (Afanasiev, 1942) to develop serious attacks.
As said earlier, it is almost impossible to determine if there will be damping-off in a field. This test could however prove to give a good indication of the condition of the sugar beet field to be. If sugar beets are grown too often in the same field, attacks will come sooner or later.

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# SURVIVAL OF *HETEROBASIDION ANNOSUM* IN DIFFERENT TYPES OF FOREST SOILS

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Summary. The goal was to evaluate the influence of the microflora on the development of *Heterobasidion annosum* inside logs buried in different forest soils. Results have shown that over a 12 and 30 month period the development of the *H. annosum* has been more extensive in the ex-pastureland soil than in the beech-wood or fir-wood soil.

## Introduction

*Heterobasidion annosum* (Fr.) Bref. is a serious parasite which particularly affects pure stands of conifers. The very heavy tree mortality that sometimes occurs in the first years after planting in soils not previously forested has often been linked to the previous use of the soil (stand history).

As part of a research programme for the biological control of *H.annosum* it was decided to investigate the development and persistence of the fungus in wood material buried in different soils with relation to the different microflora of the soil.

## Materials and methods

In autumn 1988, eight silver-fir logs, 50cm long and 10-15 cm in diameter, were buried at a depth of 20 cm in beech-wood soil,fir-wood soil and ex-pastureland soil, in two localities in the Tuscan Apennines (Acquerino, near Pistoia and Vallombrosa, near Florence). In each of the three soils at each locality six logs were inoculated on both cut surfaces with a *H.annosum* conidial suspension (10 ml with 5 x $10^4$ conidia/ml), and the remaining two logs were the controls.

In autumn 1989, after one year of interment, half the logs were recovered; the remaining logs were dug up 1½ years after that, in spring 1991. In both cases, the logs were stripped of their bark, and cut transversely into 4-5 cm thick disks. A thin strip of wood was then excised along a diameter on each side of each disk. These strips were placed on malt agar (MA) and malt agar benomyl (MAB) to isolate the fungi. The disks were stored in a humidity chamber.

The degree of *H. annosum* growth through the wood was quantified on the basis of the following variables: 1. length of wood strip colonised; and 2. disk surface covered by conidiophores (measured with a planimeter). *H. annosum* growth data were subjected to ANOVA and the mean values compared with LSD test ($P \geq 0.01\%$; $P \geq 0.05\%$).

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Tab. 1. Silver-fir interred logs. Percent of disk surface (cm ) covered by H. annosum conidiophores. Data obtained after 12 and 30 months. Each value is the average of 9 measurements.

| Type of soil | Locality: Vallombrosa 12 m. | 30 m. | Acquerino 12 m. | 30 m. | Vall. + Acq. 12 m. | 30 m. |
|---|---|---|---|---|---|---|
| ex-pastureland | 84.8 B | 45.7 b | 73.1 B | 97.5 | 79.2 B | 71.8 |
| fir-wood soil | 55.3 A | 37.8 ab | 40.1 A | 0.0 * | 52.1 A | |
| beech-wood soil | 53.4 A | 25.1 a | 40.9 A | 20.1 | 46.2 A | 38.9 |

(*) Data related to high colonization by fast growing fungi. Cfr. Tab.3. Values in the columns with dissimilar letters vary significantly at the 5% level; capital letters 1% level.

Tab. 2. Percent of wood strip colonized (lenght in cm) by H. annosum. Data obtained after 12 and 30 months. The wood strips were placed on malt agar + benomyl.

| Type of soil | Locality: Vallombrosa 12 m. | 30 m. | Acquerino 12 m. | 30 m. | Vall. + Acq. 12 m. | 30 m. |
|---|---|---|---|---|---|---|
| ex-pastureland | 52.9 b | 81.7 b | 67.3 b | 100.0 | 60.2 c | 90.8 |
| fir-wood soil | 47.2 b | 69.8 a | 41.8 a | 3.7 * | 44.5 b | |
| beech-wood soil | 31.0 a | 31.6 a | 39.2 a | 39.1 | 35.4 a | 35.3 |

(*) Data related to high colonization by fast growing fungi. Cfr. Tab.3. Values in the columns with dissimilar letters vary significantly at the 5% level.

Tab. 3. Locality Acquerino. Silver-fir logs examined after 30 months in soil. Percent of wood strip (cm) colonized by fungi. H.annosum (H.a), Trichoderma spp.(Tr.) and other fungi (o.f.).

| Type of soil | Malt-agar + benomyl % H.a | % Tr. | % O.f. | Malt-agar % H.a | % Tr. | % O.f |
|---|---|---|---|---|---|---|
| ex-pastureland | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| fir-wood soil | 3.0 | 3.4 | 82.1 | 1.2 | 16.9 | 70.1 |
| beech-wood soil | 33.8 | 0.5 | 46.6 | 29.3 | 24.6 | 32.2 |

The bacteria were isolated and counted by taking sample wood chips from the most internal part of each log. These were then weighed and soaked in 1 ml of sterilized water. The water was then streaked over the petri dish on PCA (Plate Count Agar).

## Results and discussion

After the initial year of interment, H.annosum was more frequent in the logs that had been buried in pastureland: 60.2% of strip length and 79.2% of disk surface. These values differed significantly from those for beech-wood soils (35.4 and 46.2%) and fir-wood soils (44.5 and 52.1%). After a further 1½ years in the ground, the relative proportions of H.annosum growth in the three types of soil had not changed: H.annosum growth was greater in ex-pastureland soil than in the true forest soils (Tab.1 and Tab. 2)

Various fungi which impede the H. annosum have been observed in the forest soils; among these were the Trichoderma spp. and particularly the Armillaria spp. as regards the beech-wood ( c.a 30% of the isolaments). (Tab.3)

Preliminary observations show evidence of bacteria which probably serve to impede the growth of H. annosum throughout the logs. The quantity of bacteria detected per gram of wood was, less in the ex-pastureland soil (2.9 $x10^5$) than in fir-wood soil ($3.5x10^5$) and the beech-wood soil ($1.1x10^6$).

The pH in the soil did not seem to impact the growth of H. annosum . In both the localities a greater quantity of H. annosum was found in the meadow than in the other areas. In the Acquerino locality the pH values of the three areas were quite similar (ex-pastureland 3.8; fir-wood 4.1; beech-wood 3.9), at Vallombrosa, however, the pH values were different (ex-pastureland 7.1; fir-wood 5.2; beech-wood 4.3).

Results show that the microflora in the forest soils (Alexander, et al. 1975; Srago and Cobb, 1974) help to impede the development of H. annosum especially in the interred wood as evidenced by this test. When the conifers are planted in the ex-pastureland, the pathogen could diffuse more easily due to the scarcity of microflora antagonistic toward the H. annosum.

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FACTORS INVOLVED IN REPLANT PROBLEMS OF ASPARAGUS

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## Introduction

Replant problems in asparagus are well-known in every region of the world where asparagus is grown. The cause of these problems is not sufficiently known for the asparagus fields in The Netherlands. The causal factor is very persistent: yield reductions occur even on fields where production was stopped 15-25 years ago.
The present investigations into the cause were focussed on the role of root residues as a source of autotoxins and on that of soilborne fungal pathogens.

## Results

### Autotoxicity

Root residues of asparagus decompose extremely slow in soil: ten years after asparagus production was stopped an amount of 400-1100 kg/ha (dry weight) was still present. The presence of autotoxic compounds was studied in a bio-assay in which asparagus seeds were germinated on filterpaper wetted with an extract of root tissue or an assay in which germinated seeds were grown in test tubes with perlite wetted with the extracts.
Both tests showed that an extract of roots of living plants as well as an extract of at least ten-years-old root residues contain autotoxins. Autotoxicity of the extracts was not destroyed after autoclaving at 120°C during 30 minutes.
The autotoxins were also active in soil: sterilized roots added to soil in high amounts (20 g/l, which is about ten times more than what is present one year after the production is stopped) inhibited growth, non-sterilized roots were even more inhibitory (Fig. 1).

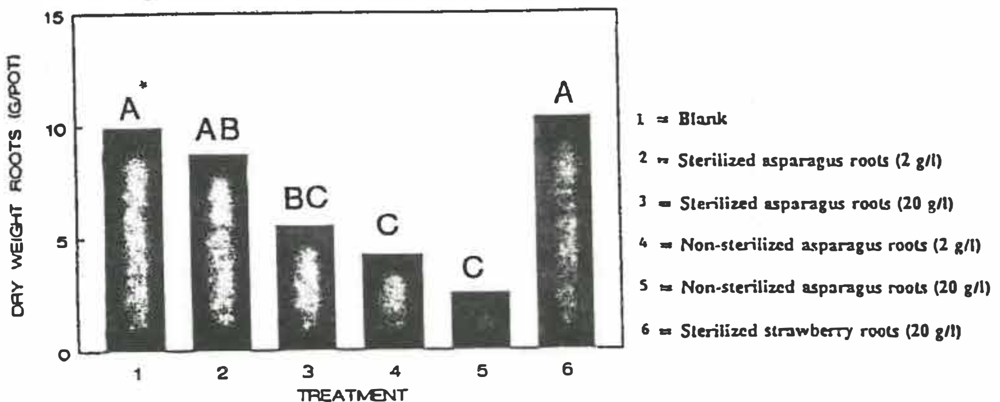


Figure 1. Effect of asparagus and strawberry root material added to field soil. on root growth of asparagus plants.

Soilborne fungal pathogens

Roots and stems from plants with foot and root rot or retarded growth were collected from five fields with a young crop, five fields with an old crop and five replanted fields. Pathogenicity of fungi from the roots and stems was tested under sterile conditions using asparagus plants on Knop's agar. Frequently isolated pathogens were *Fusarium oxysporum*, *F. culmorum*, *Penicillium* sp. and *Phialophora malorum*. From replanted fields the same pathogens were isolated (in the same relative frequencies) as in fields with a first crop.

In pot experiments under greenhouse conditions only *F. oxysporum* and *F. culmorum* reduced growth. *F. oxysporum* 'f.sp. *asparagi*' is a well-known pathogen of asparagus, causing foot and root rot and held responsible for degeneration of asparagus crops planted on fresh soil (Grogan and Kimble, 1959).

Pot experiments

By means of pot experiments it was tried to get more insight in the nature of the causal factor and to estimate the effects of autotoxins and pathogenic fungi.

The first question was if it was possible to reproduce replant problems in pots. Therefore growth of asparagus plants was compared on fresh and asparagus soil. Results are given in Table 1. On asparagus soil, root weight and number of healthy feeder roots were lower and lesions caused by *F. oxysporum* were present. These results correspond with those obtained by Huiskamp and Poll (1990).

Table 1. Root yield of asparagus plants grown for 13 weeks in soils with or without an asparagus cropping history.

| | Dry weight roots (g/pot) | Healthy feeder roots (1-10) | Lesions (0-5) |
|---|---|---|---|
| Fresh soil | 9.4 a* | 8.2 a | 0.0 a |
| Asparagus soil | 5.0 b | 2.6 b | 3.0 b |

The inhibitory factor built up very fast in soil cropped with asparagus for different periods in the greenhouse (Table 2).

Table 2. Root yield of asparagus plants grown for 13 weeks in soils with a different asparagus cropping history.

| Asparagus cropping | Dry weight roots (g/pot) | Healthy feeder roots (1-10) | Lesions (0-4) |
|---|---|---|---|
| None | 10.1 a* | 8.5 a | 1.0 a |
| 3 months | 5.5 b | 2.4 b | 3.9 b |
| 15 months | 3.6 c | 2.1 b | 4.0 b |

In an experiment in which plants were grown in mixtures of fresh and asparagus soil it was shown that the inhibitory factor spread actively through soil (Table 3).

Table 3. Root yield of asparagus plants grown for 13 weeks in mixtures of fresh (F) and asparagus (A) soil.

| Mixture | Dry weight roots (g/pot) | Healthy feeder roots (1-10) | Lesions (0-4) |
|---|---|---|---|
| 100% A | 7.8 a* | 4.4 a | 2.3 a |
| 80% A + 20% F | 7.3 a | 3.4 a | 2.9 a |
| 50% A + 50% F | 7.8 a | 3.9 a | 2.7 a |
| 20% A + 80% F | 6.4 a | 4.3 a | 2.1 a |
| 100% F | 11.9 a | 7.0 b | 1.1 b |

Heat treatment of soil at 60°C during 30 minutes eliminated the causal factor (Table 4), which excludes a direct inhibitory effect of autotoxins as the main cause, because the toxins are heat-stable and are not destroyed by heat treatment at 60°C.
In all pot experiments a reduced root weight was correlated with presence of lesions caused by *F. oxysporum*.

Table 4. Effect of heat treatment of soil on root weight (g dw/pot) of asparagus plants grown for 13 weeks.

| Soil type | Treatment | |
|---|---|---|
| | Untreated | 60°C, 30 min. |
| Fresh soil | 9.4 a* | 10.7 a |
| Asparagus soil | 5.0 b | 8.9 a |

* For the figure and all the tables: means with a letter in common are not significantly different (Tukey range test, P=0.05).

## Conclusions

- Autotoxic products from asparagus roots are present in soil till at least ten years after asparagus production was stopped.
- Direct growth inhibition by these products is not an important factor in the field. Indirect effects are under study.
- *Fusarium oxysporum* 'f.sp. *asparagi*' plays a major role in replant problems in The Netherlands.

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# GENETIC IMPROVEMENT OF ANTAGONISTS

# METHODS OF GENETIC MANIPULATION FOR THE PRODUCTION OF IMPROVED BIOPROTECTANT FUNGI

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## Introduction.

Successful biological control requires three general components, i.e. a highly effective biocontrol strain, a method of delivery that is conducive to the bioprotectant and gives it a competitive advantage relative to other microflora, and a method of production that permits production of appropriate propagules with appropriate physiological properties for biocontrol (Jin et al. 1991; Harman 1990). This Ms will summarize methods for the production of superior strains of biocontrol fungi,with an emphasis on fungi in genera *Trichoderma* and *Gliocladium*. Another recent review (Hayes 1991) provides details on some of the procedures described herein.

## Methods for selection of improved strains

Production of homokaryons - Cells of *Trichoderma* and *Gliocladium* spp. contain many nuclei. Wild strains as they are isolated may contain nuclei of different biotypes, some of which may be more effective in biocontrol than others, or than the original heterokaryotic strain. Conidia of these fungi are homokaryotic (Stasz et al. 1988a), and so progeny derived from single nuclei may represent improved biocontrol types. Strains derived from single spores also may be more stable, since different nuclear types segregate during growth of these fungi (Stasz et al. 1988a).

Chemical and physical mutation - Mutant strains induced by either chemical or physical (e.g. irradiation) means may include strains with improved biocontrol ability. For example, mutant strains resistant to benomyl were more effective than the wild-type original strains in suppressing a number of diseases (Papavizas et al. 1982), while other strain were more able than the original strains to colonize roots (i.e. to be rhizosphere competent) (Ahmad and Baker, 1987).

In addition to the direct production of improved strains, mutants may be useful in elucidation of mechanisms. Mutant strains may be deficient in production of a metabolite suspected to be involved in biocontrol, may produce additional related metabolites, or may produce higher levels of the metabolite in question (Howell and Stipanovic 1983; Knowles et al. 1988; Papavizas et al. 1982). Such strains are useful in quantitation of the effects of particular metabolites on biocontrol effectiveness. However, chemical and physical mutation is likely to induce changes in the genome in sites other than the ones of interest, and these additional changes may cause major or more subtle changes in the strains. Therefore, results of mutation studies must be interpreted with care.

Sexual genetic recombination - Strains of biocontrol fungi may differ in their biocontrol ability,(Lumsden and Locke 1989; Smith et al. 1990) and different strains may have differing and complementary capabilities. Therefore, strains that combine the desirable genetic properties of two or more strains might provide superior biocontrol strains. However, such genetic recombinants are difficult to obtain for *Trichoderma* and *Gliocladium* spp. since the sexual stage is not known for most strains of interest in biocontrol.

Protoplast fusion - Since sexual genetic recombination is difficult to obtain, research groups have attempted to obtain asexual genetic recombination through the process of parasexuality using protoplast fusion (Harman and Hayes 1991; Pe'er and Chet 1990; Stasz and Harman 1990). For additional information on this process, the reader is referred to the book on general protoplast technology in fungi (Peberdy and Ferenczy 1985) and for specific information on protoplast fusion in *Trichoderma*, to two recent reviews (Harman and Hayes 1991; Harman and Stasz 1991). In the process of parasexuality, cell fusion (plasmogamy) occurs to give rise to a heterokaryon. Within these heterokaryons, nuclei may occasionally fuse to form a diploid, and diploid nuclei may then lose some chromosomes to give rise to recombinant haploid or aneuploid nuclei (Pontecorvo 1956). In the process of protoplast fusion, single cells bounded only by a membrane (protoplasts) are produced through the action of cell wall degrading enzymes. Protoplasts of dissimilar strains are fused, usually through the addition of polyethylene glycol. Parental strains usually are prepared that contain complementary genetic markers, e.g. toxicant resistance or auxotrophy, and progeny strains with ability to grow on appropriate selective media are chosen (Harman and Stasz 1991). Within *Trichoderma*, progeny arising from fusions between dissimilar parents frequently grow slowly and may be unstable. Such strains typically sector to give rise to a wide range of strains differing in nutritional requirements, morphology, growth rate, and abundance of conidia (Harman and Hayes 1991; Stasz and Harman 1990).

This large variation can be used as a source of improved biocontrol types. Strains of *Trichoderma harzianum* improved in biocontrol efficacy and rhizosphere competence have been obtained by this process (Harman et al. 1989). One of these has been the subject of substantial industrial interest, and was recently registered by the US Environmental Protection Agency for use in field applications. However, improved strains comprise only a small percentage of the total numbers of progeny obtained by protoplast fusion, necessitating an extensive screening effort (Harman and Hayes 1991).

Genetic analysis of protoplast fusion progeny

It is useful to know the genetic nature of strains, including progeny arising from protoplast fusion. Progeny obtained are by definition heterokaryotic, and so knowledge of the relative abundance of nuclei of various kinds is useful. Isolation of

subprogeny derived from single spores can provide this information. At least some progeny appear to be strongly imbalanced heterokaryons, with nuclei of one parental type outnumbering the other by several orders of magnitude (Stasz et al. 1988a).

Isozyme analysis provides additional information concerning the genetic nature of protoplast fusion progeny. Strains of *Trichoderma* and *Gliocladium* are very diverse genetically (Stasz et al. 1989), and a series of isozyme assays that clearly and reliably distinguish between strains of these fungi were developed (Stasz et al. 1988b). Using these assays it first was possible to determine that parental strains of these fungi were homozygous for essentially all enzymes tested, and thus these fungi are presumably haploid (Stasz et al. 1988b). Second, extensive analysis of progeny from several separate fusions revealed that there was no recombination of any isozyme character. These results strongly indicated that, while protoplast fusion in *Trichoderma* gives rise to great diversity of progeny, that this occurs in the absence of recombination of characters, and therefore in the absence of parasexuality (Stasz and Harman, 1990).

Pulsed field electrophoresis provides another tool to examine the genetic nature of protoplast fusion progeny. This technique makes it possible to resolve intact fungal chromosomes (Mills and McCluskey 1990). Since parasexuality would be expected to give rise to strains in which some chromosomes arose from one parent and other chromosomes from the other parent, it should be possible to determine whether progeny strains contain a mixture of chromosomes, assuming that the chromosome profiles of parental strains differ. We have determined that there is great diversity among chromosomes of strains even within a species. Both chromosome size and number differ markedly among strains. However, at least in one protoplast fusion progeny with excellent biocontrol efficacy, chromosomes are the same as that of one parent (Hayes et al. unpublished). Again, parasexuality seems absent.

Restriction fragment length polymorphism (RFLP) analysis can be used to examine the nature of both mitochondrial and nuclear genomes. Protoplast fusion, since entire cells are fused, would be expected to contain nuclei and mitochondria from both parents. Limited analysis of progeny indicates that both the mitochondrial and the nuclear genomes of strains are derived from the parental strain indicated by isozyme electrophoresis (Hayes and Harman, unpublished). Again, parasexuality seems absent. We hypothesize a mechanism whereby only small portions of the genome are recombined in a fashion perhaps somewhat analogous to the incorporation of transposons or plasmids into nuclear DNA (Harman and Hayes 1991).

Together, these data indicate that protoplast fusion gives rise to extreme variability among progeny. It does not, however, produce true asexual hybrids in *Trichoderma*, and therefore cannot be used to recombine useful parental traits. It does, however, when combined with effective screening procedures, give rise to excellent biocontrol strains, including ones that are superior to either parent (Harman and Hayes 1991; Harman et al. 1989).

Transformation

Transformation is a process whereby small pieces of DNA from any organism can be integrated and expressed in biocontrol fungi, or any other organism (Hayes 1991). Fincham (1989) has reviewed transformation of fungi. Plasmids are available for efficient transformation of many fungi. The most efficient plasmid for transforming *Trichoderma* that we have used is that constructed and described by Herrera-Estrella, et al. (Herrera-Estrella et al. 1990). The physical process most frequently used to insert plasmids is identical to that involved in protoplast fusion, except that protoplasts of only one strain are mixed with plasmids and then fused. Alternatively, other systems, including the biolistic (gene gun) system, in which particles containing transforming DNA are physically shot into cells may also be possible (Sanford 1990). After transformation, it is necessary to isolate single spore transformants to obviate the presence of nontransformed nuclei and also to do Southern analysis to demonstrate the presence of the transformed DNA within the nucleus of the putative transformant. In this latter procedure, DNA is isolated from the transformed strain, cut with appropriate restriction enzymes, subjected to electrophoresis, transferred to a solid support and then probed with labelled DNA prepared from the plasmid used for transformation.

Several separate strategies are possible with transformation. First, genes useful in biocontrol may be isolated from any source and inserted into biocontrol fungi. In this way any useful gene may be inserted and expressed and the biocontrol agent thereby made more effective. Second, transformation results in the insertion of DNA into the genome and thereby disrupts the genetic sequence at the point of insertion. Therefore, transformation may be used to produce mutants much more precisely than chemical or physical mutagenesis and without the likelihood of multiple mutations in the progeny strain. This process of insertional mutation may be made more efficient if portions of the gene in question are identified and available since homology may favor insertion of the sequence in a precise location. Thus either insertional mutation or homologous insertion may result in mutants deficient in a specific character. These mutants may be very useful in quantification of the role of a specific genetic sequence in biocontrol. In addition, if the genetic sequence in question is available, it may also be added back to the strain to restore the missing function. In this way, a molecular version of Koch's Postulates is possible for biocontrol studies. Thomashow et al.(1990) have used such an approach to study the role of phenazine antibiotics in biocontrol of *Gaeumannomces graminis* var. *tritici* by *Pseudomonas fluorescens,* but insofar as we are aware no analogous studies have been done with biocontrol fungi.

Transformation, therefore, is a powerful tool for strain improvement and genetic studies of biocontrol fungi. Since most plasmids available confer resistance to an antibiotic, it can be used to mark strains to follow in nature. However, it cannot be

readily used to add complex traits, or products requiring multiple steps to form the product of interest, e.g. an antibiotic. It is most useful for studying or adding specific proteins. In addition, transformants may lose fitness (Keller et al. 1990) and this aspect must be considered when using transformation for mechanistic or tracking studies. An additional limitation is the current lack of genes for biocontrol and strain enhancement.

Isolation of useful genes from biocontrol fungi

Effective biocontrol agents must be storehouses of useful genes for control of plant diseases. Such genes could be isolated from these fungi and utilized either as genetic sequences to confer resistance in plants to pathogens or for addition to other microorganisms to enhance their biocontrol efficacy. Alternatively some gene products might be used directly in agriculture as a topical application.

Chitinases are among the most attractive of these gene products. Chitin is a structural component of many fungi, including plant pathogens, and of insect pests of higher plants. However it is not found in plants or mammals, so use of these enzymes should be relatively safe. In fact, chitinases are among the enzymes produced as defense mechanisms when plants are attacked by plant pathogens (Legrand et al. 1987; Roby and Esquerre-Tugaye 1987). While this group of enzymes has been suggested frequently as a factor in biocontrol by *Trichoderma* spp., chitinases from these fungi are poorly characterized.

We have examined the chitinases produced by *Trichoderma harzianum* strain P1. This fungus produces many separate chtinases, with at least three separate kinds of activity. Some of the enzymes are N-acetylglucosaminidase (nagase) and presumably cleave monomers from the end of the chitin polymer, others cleave the dimer (chitobiases), and still others cleave the polymer randomly (endochitinases). An endochitinase has been purified to homogeneity and a biase has been prepared that contains only a low level of nagase and one other protein. These enzymes are effective in inhibiting chitin-containing fungi. We are preparing cDNA libraries from these fungi and expect that genes from this fungus will be useful additions to the limited number of genes available for enhancement of biocontrol agents and to confer resistance to higher plants.

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STRATEGIES FOR THE DEVELOPMENT OF AN EXPRESSION SYSTEM FOR THE BIOCONTROLLING AGENTS *TRICHODERMA* spp

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Summary

*Trichoderma* spp are efficient producers of extracellular proteins. Many species of this genus are also regarded as biocontrolling agents. We decided to assess and develop expression vectors for these species aiming further genetic improvement in the biocontrolling process. Vectors with regulatory signals of *Aspergillus nidulans* and *Neurospora crassa* are being tested. The 3-phosphoglycerate kinase (*pgk*) gene from *Trichoderma viride* was cloned and studies of its promoter are being performed.

## Introduction

*Trichoderma* spp are fungal species which have proven to be good biological control agents against a range of economically important aerial and soil-borne plant pathogens (Chet, 1987). Recently, transformation systems have been developed for these organisms that enable the expression of both homologous and heterologous genes (Goldman et al., 1990; Herrera-Estrella et al., 1990). Other species of *Trichoderma* have already been shown to have potential as a production organism for heterologous proteins, such as calf chymosin in *T. reesei* (Nevalainen et al., 1991) and taka-amylase in *T. viride* (Cheng et al., 1990). Since our goal is to have an expression system for the biocontrol agents *Trichoderma* spp., we have decided to assess and develop vectors for these species aiming further genetic improvement in the biocontrolling process. This could include overexpression of genes important for the biocontrol, introduction of new phenotypes, and improvement of our understanding of the secretory pathways. The main strategies we are currently investigating are: (i) the isolation and characterization of expression signals of *Trichoderma* spp and (ii) the utilization of expression and secretion signals of *A. nidulans*. Here, we report some initial observations on the functionality of heterologous expression signals in *Trichoderma* spp and the isolation and characterization of the 3-phosphoglycerate kinase (*pgk*) gene from *T. viride*.

## Results and Discussion

### 1. Heterologous expression signals

We have previously reported the genetic transformation of *Trichoderma* spp (Goldman et al., 1990; Herrera-Estrella et al., 1990). Transformation was obtained with the plasmid pAN7-1, carrying a bacterial hygromycin resistance gene as a selectable marker under the control of *A. nidulans* heterologous expression signals (Punt et al., 1987). Our data provided evidence that *A. nidulans* regulatory signals are functional in *Trichoderma* spp and, furthermore, as indicated by the resistance to hygromycin (see Table 1 *in* Herrera-Estrella et

al., 1990, the levels of expression reached are similar to those reported for *Aspergillus* (Punt et al., 1987). More recently, we have transformed *T. harzianum* with the plasmid pBT6 (Orbach et al., 1986), carrying a ß-tubulin gene from *N. crassa* (that confers resistance to the fungicide benlate) under the control of *N. crassa* regulatory signals. We have obtained transformants of *T. harzianum* showing slightly higher resistance to benlate than those reported for sensitive strains of *N. crassa*, thus indicating good levels of expression of these heterologous regulatory signals in this species (G.H. Goldman, unpublished results).

2. Isolation and characterization of the *pgk* gene
A library consisting of approximately 6- to 12-kb *T. viride* *Sau*3AI fragments cloned into pT3T7.lac was screened with two synthetic oligonucleotides probes designed and based on conserved sequences of the *pgk* genes of many different species. One clone was obtained that contained an insert of approximately 5.5 kb. This insert contained the complete coding sequence as well as the 5'- and 3'-flanking regions of this gene. The coding sequence is 1,545 nucleotides long and two introns were defined based on comparisons with other *pgk* genes. Northern hybridizations have shown that this gene has only one transcript of approximately 1.7 kb. Additionally, primer extension studies have defined three transcription initiation sites at positions -360, -361, and -362, respectively.

## Conclusions

We have shown that expression signals of *Aspergillus nidulans* and *Neurospora crassa* work in *Trichoderma* spp. The *pgk* gene from *T. viride* was isolated and characterized. This gene is controlled by a very strong constitutive promoter. This promoter as well as other heterologous expression signals will be used for further development of expression systems for the biocontrolling agents *Trichoderma* spp.

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MOLECULAR KARYOTYPE OF *TRICHODERMA* spp.

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Summary
The lack of sexual and parasexual cycles in *Trichoderma* spp in the laboratory has been a major bottleneck in the study of evolution, exchange of genetic material and characterization of the different isolates. Therefore, the importance of the use of recently developed molecular biology techniques in this field. In Laboratorium voor Genetica (Gent, Belgium) the number of chromosomes of three different *Trichoderma* species has been determined by pulse-field electrophoresis and a number of genes located in the different chromosomes by using Southern analysis. The implications of these findings in the evolutionary divergence of these three species are discussed.

## Introduction

Recently studies have been initiated in Laboratorium voor Genetica (Gent, Belgium) on the molecular analysis of the mechanism of action of the mycoparasitic fungi *Trichoderma* spp. Whereas a great deal of information is available about the molecular genetics of model filamentous fungi, such as *Aspergillus nidulans* and *Neurospora crassa*, this is not the case for the generality. Our knowledge of the structural and functional organization of chromosomal DNA in this and other organisms has long been hampered by the difficulty of fractionating large DNA molecules. The development of pulsed-field electrophoresis technology has extended the range for separation of linear double-stranded DNA molecules to about 10 megabase pairs (Orbach et al., 1988). This technique along with a refinement known as contour-clamped electric-field (CHEF) gel electrophoresis (Chu et al., 1986) has been applied to resolve the genomes of several organisms into defined chromosomal bands (Schwartz and Cantor, 1984; Chu et al., 1986; Brody and Carbon, 1989).

Thanks to this technique the so-called "electrophoretic karyotype" is now becoming available for an increasing number of fungal species. We have established conditions for the preparation of intact chromosomal DNA from *Trichoderma* spp. Resolution of six chromosomal bands of up to 6.5 megabase pairs (mbp) has been achieved as well as the localization of several genes in the corresponding chromosomes by Southern analysis.

## Results

Our initial approach for the understanding of the mechanism of action of the mycoparasitic fungi *Trichoderma* spp is the establishment of molecular biology techniques for these fungi. Among these techniques we thought it was of particular interest to use pulse-field electrophoresis to establish the molecular karyotype of the most studied *Trichoderma* species (*T. harzianum*, *T. reesei*, and *T. viride*). For this purpose we have prepared intact chromosomal DNA from the

three species above mentioned essentially as described for *A. nidulans* (Brody and Carbon, 1989). The DNA obtained was submitted to both CHEF electrophoresis using the CHEF Mapper (Bio-Rad) and to rotating electrode electrophoresis using a Rotaphor apparatus (Biometra).

For *T. harzianum* it was possible to distinguish six chromosomal bands ranging from 2.23 to 6.35 mbp using a combination of both techniques. Numbers were assigned to the different chromosomal bands in order of increasing size: I (2.23 mbp), II (4.34 mbp), III (5.34 mbp), IV (5.86 mbp), V (6.3 mbp) and VI (6.35 mbp). In a similar way we have been able to define six chromosomal bands for the cellulolytic species *T. reesei*. Following the same criterium the chromosomes of *T. reesei* are: I (3.7 mbp), II (3.95 mbp), III (5.04 mbp), IV (5.54 mbp), V (6.1 mbp) and VI (6.5 mbp). Interestingly, in this case the smallest chromosomal band observed for *T. harzianum* is not present, instead one of the larger chromosomal bands appears to have increased in size as compared to the corresponding band of *T. harzianum*. In contrast, when analyzing *T. viride* we have observed only five chromosomal bands. The chromosome nomenclature assigned in this case is as follows: I (4.7 mbp), II (5.54 mbp), III (6.02 mbp), IV (6.3 mbp) and V (6.35 mbp). This species also lacks the smallest chromosomal band and contains a larger chromosomal band that when compared with *T. harzianum* has increased in size. In addition there is the apparent lack of one complete extra chromosome in this species although we can not discard the possibility that it is comigrating with other chromosomal band. However, based on the intensity of the bands upon ethidium bromide staining this does not appear to be the case.

These observations might be useful in the elucidation of the different evolutionary stages of this species since based in isoenzymes identification it has been proposed that *T. harzianum* is the ancestral species from which *T. reesei* and *T. viride* were derived (Stasz et al., 1989). Southern blot analysis using as probes a series of genes recently cloned in our laboratory allowed the localization of this genes in different chromosomes. In this way, we have localized the imidazolglycerolphosphate dehydratase (*igh*) gene to chromosome VI in *T. harzianum* and *T. reesei* and to chromosome I in *T. viride*. The ß-tubulin-encoding gene (*ben*l) was located in chromosome V in *T. harzianum* and *T. viride* and in chromosome VI in *T. reesei*. The phosphoglycerate kinase (*pgk*) gene was localized in chromosome V in *T. harzianum* and *T. viride*, and in chromosome III in *T. reesei*.

The localization of other genes is still under analysis. We expect that in this way and using partial digestions of the chromosomal DNA analyzing fragments in the order of hundreds of thousands of base pairs we will be able to establish molecular "linkage groups".

## Discussion and Conclusions

Based on the electrophoretic mobility of the chromosomes of these three species, we have estimated the genome size of *T. harzianum* and *T. reesei* to be about 30 mbp. The genome size of *T. viride* would be slightly smaller, close to 29 mbp. The sizes of the individual chromosomes indicate significant variation among the different *Trichoderma* species studied. This has also been observed in another fungal genus even among different strains of the same species (Orbach et al., 1988). However, based on size conservation and gene location, our data support the previously reported phylogenetic relationship (Stasz et al., 1989). Thus, *T. harzianum* would be the ancestral species, *T. viride* would have evolved from this species through several stages giving place to the lost of part of its genome and major chromosomal rearrangements. *T. reesei* would most probably have derived from an independent branch. Further investigation is being carried out in this direction in Laboratorium voor Genetica (Gent, Belgium).

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# HYDROLYTIC EXTRACELLULAR ENZYMES OF *TRICHODERMA HARZIANUM*: SPECIFIC INDUCTION OF A BASIC PROTEINASE

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## Summary

Hydrolytic enzymes secreted by *Trichoderma harzianum* are believed to play an important role in the biocontrolling process of phytopathogenic fungi. Among these enzymes we have identified a basic proteinase (PrB-1) that is induced in the presence of either autoclaved mycelia or cell walls of phytopathogenic fungi. The proteinase has been biochemically characterized and identified as a serine proteinase. By using synthetic oligonucleotides deduced from peptide microsequencing data, we have been able to clone the corresponding cDNA from a library generated using poly(A)$^{+}$ RNA from induced mycelia. The gene encoding for PrB-1 has been mapped to chromosome 6 by Southern analysis of chromosomes separated by pulse-field electrophoresis. Northern analysis has shown that induction by cell walls occurs at the RNA level. In addition, preliminary immunofluorescence experiments indicate that this proteinase is produced only in the areas of direct physical contact with the pathogen when they are confronted *in vitro*.

## Introduction

The importance of studying *Trichoderma spp* has been shown from both the agronomic and industrial point of view. *T. viride* and *T. harzianum* have been widely used as biocontrolling agents against phytopathogenic fungi, whereas *T. reesei* is well known for its ability to produce potent cellulose-degrading enzymes. In addition, *T. reesei* and *T. viride* have been successfully used for the production of heterologous proteins (Harkki et al., 1989; Cheng et al., 1990). Therefore, studies at the biochemical and molecular level are needed.

Of major relevance is the study of the large variety of proteins secreted by these fungi, since their regulatory and secretory signals will be very useful for the development of new expression vectors. These vectors could later be used for the production of heterologous proteins and for the improvement of the biocontrolling characteristics of a given strain.

## Results and discussion

Our goals are the better understanding and improvement of the biocontrolling process. For this purpose, we focused our attention on the lytic enzymes produced by *T. harzianum* during its interaction with phytopathogenic fungi. Among the enzymes produced by the fungus in this process we have detected ß(1,3)-glucanase, chitinases, and proteinases. Here, we describe our studies on the pattern of secreted proteinases when the fungus is grown with different carbon sources,

including autoclaved mycelia and cell walls of target fungi. Under these conditions, we have been able to identify two proteinases, one neutral and one alkaline. The alkaline proteinase was present only when the mycelium was cultured in the presence of either (i) autoclaved mycelia of different phytopathogens, (ii) *R. solani* cell walls, or (iii) colloidal chitin. It was not present if glucose was added together with the former carbon sources, indicating that the secretion of the alkaline proteinase is repressed. On the other hand, the neutral proteinase was present only when glucose was added to the media. These results indicate that the neutral proteinase is not inducible by phytopathogens, whereas the alkaline proteinase is inducible by a common factor present in the autoclaved mycelia, cell walls, and chitin.

Detailed studies of the production of the alkaline proteinase have revealed that it is not released into the culture media when the fungus was grown in the presence of glucose, even when *R. solani* cell walls or colloidal chitin were present. However, when glucose was omitted from the culture media and *R. solani* cell walls or colloidal chitin were present, the alkaline proteinase activity was released into the culture media after 16 hours and reached a maximum after 72 hours of culture. In medium without glucose or any alternative carbon source, no alkaline proteinase activity was detected in the culture medium even after four days of culture, ruling out the possibility that the induction is simply due to starvation. It is noteworthy that albumin and gelatin failed to induce the secretion of this enzyme. This type of specificity is consistent with an enzyme only produced to attack the protein layer present in many fungal species.

The proteinase was purified to homogeneity (as indicated by SDS/PAGE) by traditional techniques. This enzyme uses substrates for chymotrypsin, and is inhibited by PMSF. The later results indicate that it is a serine proteinase. Its molecular mass was estimated to be 30 kDa and its pI 9.6; it is active at a broad pH range with a maximum between 8 and 11. Internal peptides of the protein were obtained and three of them were microsequenced. The deduced oligonucleotides (31 to 41 bp) were synthesized and used for screening as described later.

A *T. harzianum* cDNA library was constructed, using poly(A)$^{+}$ RNA isolated from *T. harzianum* grown for one day in the presence of *R. solani* cell walls. The library was constructed using a shuttle vector developed in Laboratorium voor Genetica (Gent, Belgium) (Goldman et al., in preparation). This library was screened with a mixture of the three synthetic oligonucleotides. Plasmid DNA of 40 positive clones was hybridized using each one of the synthetic oligonucleotides independently as a probe. Thirteen clones gave positive signal with two of the oligonucleotides. The largest open-reading frame present in the largest of these clones encodes a protein of 42.3 kDa (PrB-1). In this protein the three peptide sequences obtained by microsequencing are present. Computer analysis has shown that consensus sequence of the active site resembles a subtilisin-type serine proteinase. Comparison of the Prb-1 protein with either the Swiss or Pir protein data bank revealed homology with other subtilisin-type serine proteinases. The higher homologies were with precursors of different fungal proteinases: 57% with the *Aspergillus oryzae* alkaline proteinase precursor (Tatsumi et al., 1989), 37% with the *Tritirachium album* proteinase T and proteinase K precursors (Gunkel and Gassen, 1989), and 36% with the *Yarrowia lipolytica* extracellular proteinase (Davidow et al., 1987). The presence of the peptides, consensus sequence, the homology data, the facts that this proteinase is secreted, and that these proteinases are synthesized as zymogens indicate that the PrB-1 is a preproform of the alkaline proteinase previously observed. The first 24 amino acids of this protein resemble a signal peptide required for targeting of nascent proteins to the endoplasmic reticulum, since its amino-terminal end is positively charged and is followed by a core of hydrophobic amino acids. Based on this data we suggest that the mature protein starts at amino acid 112 of the preproprotein. Making this assumption the mature protein should have a molecular mass of 27.6 kDa and a pI 9.7. These results are in line

with those obtained with the isolated alkaline proteinase. Experiments of expression of this clone in *Saccharomyces cerevisiae* are being carried out.

In order to determine whether the induction observed is at the mRNA or protein level, Northern analysis were carried out. Total RNA isolated from mycelia grown in the presence of *R. solani* cell walls with or without glucose were harvested at different times after induction. The RNA was electrophoresed, Northern blotted and hybridized with either the Pbr-1 cDNA or a *T. viride* ß-tubulin cDNA. Messenger RNA corresponding to the alkaline proteinase was detected only in samples from *T. harzianum* grown in the presence of cell walls without glucose, whereas ß-tubulin transcript was detected in all samples. These data indicate that the increase in the amount of secreted proteinase is due to an induction at the RNA level.

On the other hand, in order to study the role and specificity of this enzyme in the biocontrolling process, immunofluorescent microscopy was performed, using an antibody raised against the alkaline proteinase. Preliminary data indicate that the immunoreaction is located only at those places in which direct contact between *R. solani* and *T. harzianum* takes places. No reaction was detected using either fungi independently.

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# PROTOPLAST FUSION IN ANTAGONISTIC FUSARIUM SPP.*

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**Summary**
Protoplast fusion was applied for the selection of intra- and interstrain hybrids of antagonistic *Fusarium* spp., active against *F.oxysporum* f.sp. *dianthi*. Auxotrophic, color and benomyl-resistant mutants were obtained from antagonistic *Fusarium* isolates after exposure to uv radiations or N-nitrosoguanidine. Protoplasts from these strains were produced by incubating thalli or germinated conidia for 3-20 h at 30°C with different combinations of chitinase and β-glucuronidase. Six intra- and interstrain crosses were performed and several fusants selected by the combination of different parental markers.

## Introduction

Soil suppressiveness to *Fusarium oxysporum* f.sp. *dianthi* in France and Italy was related to the presence of high populations of antagonistic *Fusarium* spp. (Garibaldi and Gullino, 1987). Efforts have been devoted to screening antagonistic isolates from *Fusarium*-suppressive soils and to improving their activity by physical and chemical mutation. Protoplast fusion (Peberdy, 1979) could also represent a useful tool for manipulating *Fusarium* spp. in order to select new biotypes to be tested against *F.dianthi*.

## Materials and Methods

*Fusarium* spp. strains 141, 233, 245 and 251, isolated from the rhizosphere of carnations grown in suppressive soils of Liguria (Italy), were exposed to uv or N-nitrosoguanidine and several auxotrophic, color ("C") and benomyl-resistant ("RB") mutants were obtained. Protoplasts were produced from such mutants by treating 7 days-old mycelia or germinated conidia with different combinations of β-glucuronidase (2.0-7.5%) and chitinase (0.25-1 mg/ml) in 0.6 M KCl. Incubation at 30°C varied from 3 to 20 h on a rotary skaker (70 rpm). A 30 min pre-treatment (30°C) with 5.0 mM EDTA + 0.2% β-mercaptoethanol, pH 4.5 was also used in order to increase protoplast formation from some strains. After filtration through a 15 μm nylon filter protoplasts from two parental strains were washed with 0.6 M KCl, mixed in the same ratio, centrifuged in 0.8 M sucrose gradient for 10 min at 800 rpm, resuspended in the same volume of 0.6 M KCl, 25% polyethylene glycol (PEG 3,350) and 1.32% $CaCl_2$ and incubated for 30 min at room temperature. The fusion mixture was then washed with 0.6 M KCl, suspended in 0.8 M sucrose and plated on mineral medium (MM) or on a 0.8 M sucrose-enriched malt agar medium amended with benomyl (10 μg/ml). Fusants from complementary auxotrophic parental strains grew on MM, while the combination of color and benomyl resistance allowed the isolation of fusants from crosses between "C" and "RB" mutants.

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Table 1. Protoplast preparation from mycelium (M) or germinated conidia (C) of _Fusarium_ spp.

| Strain | Source | β-ME+ EDTA* | Enzyme concentration β-glucuronidase (μl/ml) | chitinase (μg/ml) | Incubation (h) | Protoplasts obtained ($10^4$/ml) |
|---|---|---|---|---|---|---|
| 251/3 | M | - | 20 | - | 3 | 540 |
| 251/3 S1 | M | - | 20 | - | 3 | 500 |
| 251/3 C6 | M | + | 37 | 500 | 18-20 | 16 |
| 251/3 C1 | M | - | 20 | - | 3 | 380 |
| 251/3 1500 Ile-Val- | C | - | 20 | 250 | 3 | 50 |
| 251/3 1931 Ile- | M | + | 37 | 500 | 18-20 | 220 |
| 251/3 701 Lys- | M | + | 37 | 500 | 18-20 | 1700 |
| 251/3 701.2 Lys- | M | - | 20 | - | 3 | 390 |
| 251/3 B15 Trp- | M | + | 50 | 750 | 18-20 | 270 |
| 251/3 1111 Met- | M | - | 37 | 500 | 18-20 | 12 |
| 233 wt | M | + | 37 | 500 | 18-20 | 9 |
| 233/1 C5.9 | M | - | 20 | - | 3 | 23 |
| 233/1 903 Met- | M | - | 40 | - | 3 | 41 |
| 141 2453 Met- | C | - | 75 | 1000 | 3 | 57 |
| 141 C7.2 | M | - | 20 | - | 3 | 680 |
| 245 wt | M | - | 20 | - | 3 | 43 |

*pre-treatment with 0.2% β-mercaptoethanol in 5 mM EDTA, pH 4.5

**Results and discussion**

Protoplast yield varied between $9.0 \times 10^4$ and $1.7 \times 10^7$ protoplasts/ml, depending both on the strain used and on the particular procedure adopted (Table 1). A pre-treatment with 5mM EDTA+0.2% β-mercaptoethanol was necessary for achieving protoplast release from isolate 233 wt and auxotrophic mutants 251/3 1931 Ile- and 251/3 701 Lys-. Some of the tested strains allowed adequate protoplast yields following a simple digestion of mycelia in 0.6 M KCl containing 2% β-glucuronidase for 3 h, while others required a double concentration of this enzyme for protoplast release (Table 1). Chitinase was shown to be essential in the enzymatic digestion of mutants C6, 1931 Ile-, 701 Lys-, B15 Trp-, 1111 Met- (all deriving from strain 251/3) and the strain 233 wt. Germinated conidia of mutants 251/3 1500 Ile-Val- and 141 2453 Met- released protoplasts only when exposed to both chitinase and β-glucuronidase (Table 1). These results suggest that the development of broadly applicable procedures for protoplast production seems rather difficult and each technique should be adapted to the particular strain to be processed.

Six fusions were successfully performed using auxotrophic, color or benomyl-resistant mutants as parental strains (Table 2). The number of fusants carrying both parental markers greatly varied for each fusion experiment and no correlation was found between the frequency of fusants isolated and the number of protoplasts plated after each fusion. Both complementary auxotrophy and the combination of color mutations and benomyl resistance allowed an easy recognition of hybrids. Yet, while some grade of reversion was observed in fusants from auxotrophic mutants, those deriving from color and benomyl-resistant parental strains proved to be stable even in prolonged culture in the absence of fungicide.

Table 2. Fusion experiments in antagonistic _Fusarium_ spp.

| Cross | Parental strains and markers* | Isolated fusants | and markers |
|---|---|---|---|
| I | 251/3 701 Lys- x 251/3 1931 Ile- | 13 | Lys+, Ile+ |
| II | 251/3 C1 RB x 245 wt | 28 | RB |
| III | 251/3 701 Lys- x 233/1 903 Met- | 2 | Lys+, Met+ |
| IV | 251/3 RB x 141 C7.2 | 147 | RB, C |
| V | 251/3 S1 RB x 141 C7.2 | 13 | RB, C |
| VI | 251/3 B15 Trp- x 233/1 903 Met- | 8 | Trp+, Met+ |

* C = color mutation (light orange pigmented colonies)
RB = resistance to benomyl (>10 μg/ml)

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# PRODUCT DEVELOPMENT

# PRODUCTION AND DELIVERY SYSTEMS FOR BIOCONTROL AGENTS

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## Introduction

Biological control must be dependable and reliable if it is to become an important component of plant protection. In our experience (Harman, 1991), it is essential to develop biological control systems. Such systems depend upon three critical components, i.e. a highly effective biocontrol agent, production of a high level of effective and viable propagules, and delivery systems conducive to the bioprotectant and which provide a competitive advantage to the biocontrol agent relative to other microflora. Other papers in this volume address development of wild and genetically manipulated biocontrol agents; this paper will discuss development of production and delivery systems, with the emphasis on *Trichoderma harzianum* as a biocontrol agent and seed treatment as the delivery system.

## Fermentation systems for *Trichoderma harzianum*

Biological control requires methods for the production of large amounts of biomass with specific properties (Harman, et al. 1991) as follows: (1) propagules must be of an appropriate type, (2) production must be economical, and preferably in submerged liquid fermentation (3) biomass must be preserved against microbial contamination, usually by formulating as a dry powder or otherwise obtaining low water availability (Kenney and Couch, 1981), (4) a high percentage of the propagules must be germinable and effective, and (5) biomass should have a long shelf life. It is difficult for preparations of any organism to possess all of these characteristics, but biocontrol preparations should have as many as possible of these desirable properties.

When considering *Trichoderma* spp., it is possible to choose hyphae, chlamydospores, or conidia as the basis of a biocontrol preparation. Hyphae are unlikely to be useful since they will not withstand drying. Either conidia and/or chlamydospores may be produced (Lewis and Papavizas, 1983), but we decided to produce conidia. Under favorable conditions, conidia are usually produced more abundantly than chlamydospores. However, they usually are produced in aerial environments, and therefore, media must be sought that produce conidia in submerged liquid culture. The discovery of appropriate media may be strain specific, but is the first step in the development of appropriate biomass. We found that Richard's medium with a low level of sucrose was appropriate for *T. harzianum* strain ATCC 20847. However, this medium alone produced low levels of total biomass, a deficiency that could be corrected by the addition of V8 juice ((Harman, et al., 1991). However, while conidia of this strain were produced in abundance in this medium, only a small percentage of these propagules

survived drying. This problem was alleviated lowering the water potential of the medium to a level of about -2 MPa. Dried biomass produced under these conditions was more effective in biological seed treatments of cucumbers than that from an industrial fermenter (Harman et al., 1991). Polyethylene glycol was useful in this regard, and fermentation conditions giving rise to dry preparations with a high level of viable propagules have been developed for polyethylene glycol of two different molecular weights (Harman et al., 1991; Jin et al., 1992). Biomass consisting of appropriate propagules and with good desiccation tolerance is likely to be useful in biological control.

## Delivery systems

Biological control agents usually must proliferate rapidly and become established on the place where they are applied. The first 12-24 hr after planting is a critical time for bioprotectants applied as seed treatments, and they need to grow and become established on the seed coat within this time (Hubbard, et al. 1983; Taylor and Harman, 1990). If the bioprotectant becomes established on the seed, it may not only protect the seed from seed- or soil-borne plant pathogens, but it may also colonize and protect roots if it has the genetic ability to do so.

Biological seed treatments therefore must provide a conducive milieu for the biocontrol agent to begin its growth, and also should limit growth of competitive microflora. There are several general approaches to achieve these goals. While developed for seed treatments, these concepts have validity for other delivery systems. Conceptually, the seed may be colonized by the bioprotectant before it is planted or the seed treatment may permit colonization of the seed after planting while excluding competitive microflora. General components of seed treatment systems useful in achieving effective biocontrol include materials that regulate pH to favor the organism in question, food bases specific for the bioprotectant, and toxicants, including but not limited to fungicides that enhance the range of pathogens controlled, efficacy under adverse conditions, or that limit competitive microflora (Taylor and Harman, 1990). Specific examples of some of these treatments follow.

## Solid matrix priming

Seed priming results when seeds are hydrated to a level sufficient to begin the metabolic processes of germination, but inadequate for seed sprouting. Adequate aeration is essential to prevent seed death due to anoxia. Solid matrix priming (SMP) was conceived by Kamterter, Inc. and relies upon a finely ground solid matrix to regulate water levels in the seeds. We utilized this process to enhance biological control using either *Trichoderma* spp. or *Enterobacter cloacae*. Neither organism requires as much water to proliferate as seeds do to sprout, and they grow on the seed surface during the priming process, and may increase in numbers by one order of magnitude. For *Trichoderma*, the matrix material was a lignite with a pH of 4.1, while with *E. cloacae* a

coal with a pH of 6.6 was employed. These favorable pH levels for the two organisms was necessary to achieve satisfactory results (Harman and Taylor, 1988). This process frequently resulted in dramatic improvement in biocontrol efficiency, particularly against *Pythium* spp (Harman and Taylor 1988; Harman, et al. 1989). It provides for effective colonization of the seed surface prior to planting, and employs favorable pH levels. Food bases are provided primarily by seed exudates in an environment where competitive microflora are limited.

Liquid and double coating

Liquid coating is a process in which the bioprotectant is applied to the seed in a mixture comprising itself, an appropriate sticker (adhesive), and a particulate material chosen to regulate pH to a favorable level and to provide a bulking agent(Taylor et al. 1991). This mixture is applied to the seed contained in a revolving drum with an aerosol suspension. By appropriate choice of materials and skill in application, it is possible to produce a thin, continuous coating on the seed surface. Even a thin layer (< 0.1 mm thick) can improve biocontrol efficacy (Taylor, et al., 1991). The layer provides a conducive environment to the bioprotectant, and it also excludes competitive soil microflora immediately after planting.

The coating also provides an important improvement in timing of microbial growth around the planted seed. *Pythium* spp. are among the most important seed attacking fungi, and respond very rapidly to planted seeds. Within 4-6 hours after planting, some seeds may already be infected, while *Trichoderma* spores may not germinate for 12 hr or more after planting. The coating slows the infection of seeds by *Pythium*, since they must traverse this coat as they infect from soil, and allows the bioprotectant to become active before seed infection (Taylor et al., 1991).

A modification of liquid coating is double coating; in this process *Trichoderma* is applied directly to the seed coat, and a second layer containing the particulate is applied over the bioprotectant. Spores of the biocontrol agent are in a better position to take advantage of seed exudates than when it is dispensed within the coating. This advantage may be important with seeds that have a low level of exudation.

Liquid coated or double coated seeds may also be colonized by the bioprotectant prior to planting. If freshly treated seeds are placed at 100 % relative humidity for a few days after treatment, *Trichoderma* will proliferate over the seed surface and provide a substantial improvement in biocontrol efficacy (Taylor et al., 1991).

Thus, the various derivatives of liquid coating may provide an environment permitting the bioprotectant to begin growth after planting in the absence of competitive microflora, or by the high relative humidity treatment, may permit colonization of the seed

before planting, and therefore enhance biocontrol efficacy (Taylor et al., 1991).

Application of concepts to other delivery systems

These concepts also have application to other delivery systems. For example, Lewis and Papavizas (1987) applied germlings of *T. hamatum* to soil and these were highly effective in control of *R. solani*. This process was analogous to SMP or liquid coating plus high relative humidity as a method to permit colonization of seeds prior to planting. The seed treatment procedures have an advantage to using germlings, in that seeds may be dried and stored after treatment while germlings must be used immediately.

Food bases when applied as seed treatments must be done with care. If materials stimulatory to plant pathogens as well as the bioprotectant are employed, disease may actually increase as a consequence of the treatment. Food bases must either be specific to the bioprotectant (Nelson et al., 1988) or else food bases must physically be separated from pathogens and competitive microflora, e.g. by a seed coating. Granules containing food bases have been used successfully, in this case the food base is contained within the granule and is primarily available to the biocontrol agent.(Harman and Lumsden, 1989).

Toxicants may also be employed in a variety of biological control systems. Pesticides have been widely used as an integrated biological/chemical seed treatment. The use of chemical agents may allow disease control under unfavorable edaphic conditions, for example, some strains of *Trichoderma* may be ineffective as seed treatments in cold soils. Addition of a compatible protectant such as metalaxyl may provide an effective treatment (Taylor and Harman, 1990). The use of integrated biological/chemical treatment may permit control of pathogens outside the range of the bioprotectant itself. In addition, chemicals are likely to be used under many condition where biologicals may be employed. The use of pesticide-resistant strains may permit biologicals to be used in an integrated control system employing both biological protectants and chemical pesticides (Tronsmo, 1991).

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# APPLICABILITY OF DIFFERENT FORMULATIONS OF FUNGAL ANTAGONISTS FOR THE CONTROL OF SOIL-BORNE DISEASES.

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## 1. SUMMARY:

First results from investigations on formulation and application of antagonistic fungi for the control of soil-borne diseases are presented.
Expanded Clay, Perlite, Hardwood Bark and Vermiculite as different inorganic and organic substrates were tested for their applicability to be used as growing media and carriers for the application of fungal antagonists. *Trichoderma harzianum*, *Gliocladium roseum* and *Beauveria bassiana* developed and sporulated well on the tested substrates and for each fungus the optimal method of formulation was found. Storage of the formulations resulted in different viabilities of the fungi. First results are shown concerning the biological control as well of damping-off of lettuce, cucumber and pea caused by *Rhizoctonia solani* and *Pythium ultimum* as of *Otiorrhynchus sulcatus* on juniper.

## 2. INTRODUCTION:

For the practical use of fungal antagonists to control soil-borne diseases the form and mode of application is of major importance. Such a formulation should allow the user to introduce the biocontrol-agent to soil in a concentration sufficient to reach a high level of efficiency, duration and security of the biocontrol-effects, even under unfavourable environmental conditions.
The application of fungal antagonists with special carriers, non-conducive to growth of other soil-microorganisms, permitting good development and storage of the fungi should lead to successful establishment of the biocontrol-agents in soil.
Subsequently some results from investigations on the formulation and application of *Trichoderma harzianum*, *Gliocladium roseum* and *Beauveria bassiana* for the control of *Rhizoctonia* and *Pythium* damping-off of lettuce and pea and the black vine weevil on juniper are shown.

## 3. RESULTS:

Four different organic and inorganic substrates, that can be used as aditional compounds in horticultural soils, were tested for their applicability to be used as growing media and carriers for the formulation and application of fungal antagonists:

- »» Expanded Clay (Lecadan, 2-4 mm)
- »» Perlite (Agriperl)
- »» Hardwood Bark (5-10 mm)
- »» Vermiculite (Granulation No. 3)

*T. harzianum* used as circular agar disks overgrown with mycelium showed considerable growth and sporulation on all substrates when these had first been steeped in a nutrient solution comprising 2% biomalt and then sterilized. The best development was found on Hardwood Bark (Table 1).

*G. roseum* also showed best development on Hardwood Bark, however, in this case the substrates were first sterilized and then steeped in biomalt nutrient solution (2%) mixed with a spore-suspension of *G. roseum* derived from agar plates (Table 1).
*B. bassiana* showed best development on Vermiculite when substrates were treated as described for *G. roseum* (Table 1).
Incubation time at 23°C in the dark was the same for all substrates but differing for the fungi.
After storage under various conditions different viabilities of the formulations were found (Tabel 1).
Independent from the carrier used each fungus could be reisolated from soil in greenhouse experiments under various environmental conditions till 9 month after application.

__Table 1:__ Sporulation of *T. harzianum*, *G. roseum* and *B. bassiana* on different substrates after 7 days of incubation at 23°C in the dark and viability of these formulations after drying and storing in closed glasses at room temperature.

| Substrates | Number of spores ($x 10^4$) | | | Time of survival (month) | | |
|---|---|---|---|---|---|---|
| | T. harz. | G. ros. | B. bass. | T. harz. | G. ros. | B. bass. |
| Exp. Clay | 1,8 | 1,4 | 2,9 | >12 | >12 | 4 |
| Perlite | 16,5 | 0,4 | 2,4 | >12 | 6 | 3 |
| Bark | 36,5 | 2,6 | 2,0 | >12 | >12 | 5 |
| Vermiculite | 8,2 | 2,4 | 7,5 | >12 | >12 | 6 |

*T. harzianum* and *G. roseum* applicated to soil with any of the four carriers could diminish the decrease in dry-matter-production of lettuce caused by *Rhizoctonia solani* (Fig.1).
Both, *T. harzianum* and *G. roseum* grown on Bark and applicated to soil in a concentration of 5 vol.% prophylactically could protect pea seedlings from damping-off caused by *P. ultimum* under greenhouse conditions (Fig. 2).
Damping-off of cucumber caused by *R. solani* could be prevented by the same treatment but with lower efficiency (Fig. 3).
Damages on juniper caused by larvae of *O. sulcatus* could not be prevented effectively although prophylactic application of B. bassiana grown on bark resulted in 50% mortality of larvae.

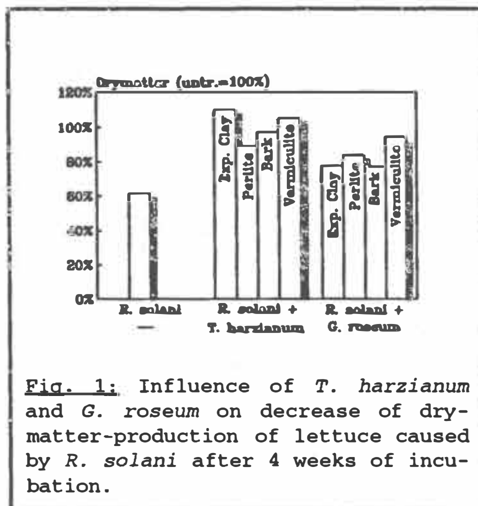


__Fig. 1:__ Influence of *T. harzianum* and *G. roseum* on decrease of dry-matter-production of lettuce caused by *R. solani* after 4 weeks of incubation.

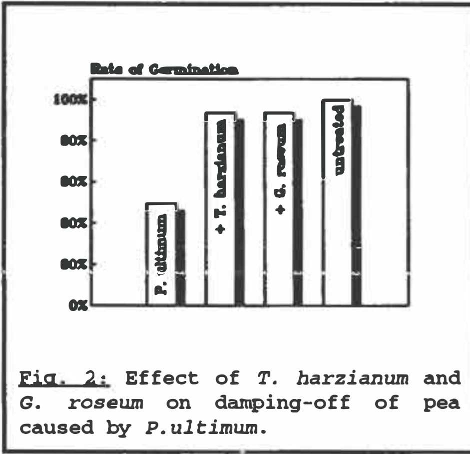


Fig. 2: Effect of *T. harzianum* and *G. roseum* on damping-off of pea caused by *P.ultimum*.

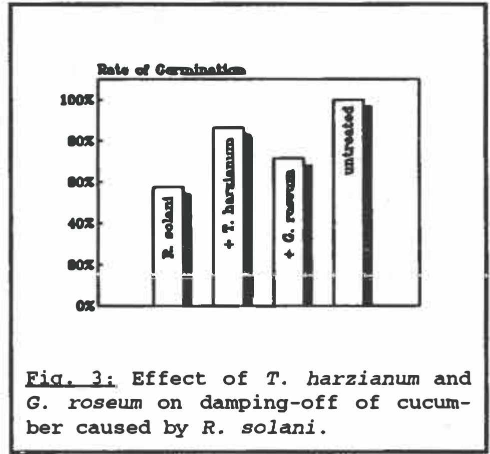


Fig. 3: Effect of *T. harzianum* and *G. roseum* on damping-off of cucumber caused by *R. solani*.

## 4. CONCLUSIONS:

The investigations until now have shown that the substrates tested can be used as growing media and carriers for the application of fungal antagonists to soil.
Further research is necessary to answer questions concerning:

- » duration of effects in soil
- » effects in different soils under various environmental conditions
- » influence of storage of the formulations on the efficiency of the biocontrol-agents

## RESEARCH ON FUNGAL BIOCONTROL AGENTS IN INDUSTRY
Illustrated by control of *Rhizoctonia solani* with *Verticillium biguttatum*

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### Introduction

Research at institutes and universities often results in new ideas on biological control methods. If an idea is to be developed into a product by a commercial company much more research is needed to bring this biocontrol agent on the market.
This publication presents one of the research projects at Koppert, the biological control of *Rhizoctonia solani* in potato by *Verticillium biguttatum*, which illustrates the steps to be taken during the process of product development, as it is done at this company.

Koppert is a commercial company which produces biological crop protection agents. The main products consist of parasitic wasps, predatory mites and insects and microbial agents, which are now marketed throughout the world.
Besides the production department Koppert has its own research and development department where new developments are constantly being sought. Research is focussed on:
- evaluation of new biocontrol agents
- mass production systems
- formulation methods
- testing in agricultural practice
- registration procedures
- quality control.

### *Rhizoctonia solani* in potato

*Rhizoctonia solani* is an important source of damage in potato crops. Damage consists not only of yield reduction by stem canker and stolon attack, but also the quality will be reduced by black scurf on the tubers.

#### Present control
Potato plants can be infected by the fungus from the seed tuber (sclerotia) or from the soil (plant debris). In a wide crop rotation programme, with a potato crop grown only once in 4-5 years, the infection from the soil is eliminated considerably. With the increased frequence of potato cropping, however, the disease has become more severe. Control of *Rhizoctonia solani* is therefore absolutely necessary. The present control of *Rhizoctonia solani* consists of tuber disinfection and soil treatment with the specific anti-*Rhizoctonia* fungicides Monceren (pencycuron) and Solacol (validamycine). A new concept is the treatment of tubers with a hyperparasite of *Rhizoctonia*, the fungus *Verticillium biguttatum* (Jager et al, 1990).

#### Biocontrol with *Verticillium biguttatum*
*Verticillium biguttatum* occurs naturally in the soil. The fungus can be found, with masses of spores as white spots visible, on sclerotia of *R. solani* on the tubers. *V. biguttatum* is able to parasitize and kill hyphae and sclerotia of *Rhizoctonia solani* and to protect young sprouts from penetration by *R. solani*.
Application of *V. biguttatum* is possible at three different stages, at which the fungus will be applied as a spore suspension:
- treatment during planting (spring treatment) which will prevent the penetration of sprouts and stolons by *R. solani*.
  Pre-germinated seed tubers should be treated. When the sprouts grow, the spores of *V. biguttatum* will be passively transported to newly formed stolons and roots.

- treatment during green crop harvesting which will kill sclerotia on the tubers and will prevent the formation of new sclerotia.
  The common farm practice, chemical haulm destruction to prevent transfer of virus diseases to the tubers, aggrevates the formation of sclerotia on young tubers. Green crop harvesting is a new harvest method that doesn't stimulate the formation of sclerotia. Moreover it gives the possibility to apply *V. biguttatum* at the first lifting, just before most of the black scurf is being formed. During the first lifting foliage is being removed from the field and the tubers are covered again with soil. The final harvest will take place after ten or more days. This harvest method is still in research.
- treatment prior to storage which will kill sclerotia present on the seed tubers during storage. Favorable conditions for *V. biguttatum* (temperature of 18 °C, relative humidity 100%) should be maintained for three weeks.

Difficulties concerning biocontrol with *Verticillium biguttatum*

* Efficacy under various conditions
  *V. biguttatum* requires a relatively high temperature and a high humidity. A high humidity is necessary to prevent the drying up of the inoculation material, which will cause most of the spores to die. Minimum temperature for growth is 12 °C, while *R. solani* starts growing at a temperature of 1 °C. Good control is achieved between 15 and 20 °C.
  Soil temperatures in spring can be 8 to 12 °C, which is too low for a good control in the field. During storage a high temperature and humidity can induce other potato diseases and sprouting of the tubers.
* Competitiveness with present methods of control
  - Efficacy
    Field experiments showed that integrated control with *V. biguttatum* and 1/4 of the normal dose Monceren can be as effective as chemical control with 1/1 Monceren (Jager et al, 1990). In some fields, however, biological control failed. The efficacy of a treatment during planting is variable. Treatment of *V. biguttatum* during storage and green crop harvesting gives promising results.
  - Application method
    Farmers prefer not to change their application methods. Treatment particularly during green crop harvesting, implicates a change of the harvest method with different equipment.
  - Price
    For farmers to accept the new control method the price should not be much higher than the price of the present control.
* Compatibility with control of other potato diseases
  Besides *Rhizoctonia solani*, other diseases play an important role in the potato growing: *Phoma exigua*, *Erwinia* spp. (bacterial diseases), *Fusarium* spp. (dry rot), *Helminthosporium solani* (silver scurf) and *Phytophtora infestans* (potato blight). To control these diseases fungicides should be applied during green crop harvesting or before storage. Integration of biocontrol and chemical control is hardly or not possible because *V. biguttatum* is susceptible to the fungicides used to control these diseases.

These problems are being studied further. Research on the biocontrol of *Rhizoctonia solani* is carried out by a working-group representing various institutes and Koppert.

Research on this project by Koppert

At this moment Koppert is evaluating the possibilities for marketing *Verticillium biguttatum* as a commercial product. The following topics are explored:

- market
  The market should be sufficiently large enough to justify the expense of research, production and registration. Market research is carried out to explore the potential market. Results show that about $170 per hectare is spent on fungicides to control *Rhizoctonia solani*. Farmers show a positive attitude towards the new biological product, provided that the price and efficacy agrees with the existing chemical control.

- mass production
  The biocontrol agent must be produced in large quantities, at economic prices, to be able to deliver sufficient quantities to the users. A feasible production medium and fermentation technique are being developed.
- drying
  A dry product compared to a liquid product has the advantages of being convenient for storage and transport. Drying of the spores prolongs the shelf life of the product. A drying method which maintains the viability of a high percentage of the spores is being searched for.
- formulation
  Formulation is required to stabilize the product during storage and to facilitate the delivery onto the tubers. Besides other additives, a sticking agent will be needed to stick the spores on the tubers.
- efficacy
  More field experiments are being carried out to prove the efficacy of the product, especially the dose-effect relationship is being examined.
- registration procedure
  Data on efficacy and toxicity have to be collated and presented to the authorities. The product should be effective under various conditions, and competitive to the existing control method. The biocontrol agent must be safe (non-toxic and non-pathogenic) for non-target organisms, including man. Financially the registration procedure is a very uncertain factor. Untill now there are no clear guidelines for registration of microbial crop protection products. Costs for acute toxicity tests alone are at least $100.000, with the possibility that sub-acute tests will have to be performed at a later stage (costs over $500.000). The registration with the authorities takes a minimum of two years.

Economic viability of commercial production of *Verticillium biguttatum*

The profitability of the prodution of *V. biguttatum* depends on:
- costs of research, production, formulation, registration
- spore yield per production unit and per time unit
- spore concentration needed for application
- reliable results under various conditions
- price of the product
- total market size.

## Conclusions

A new biocontrol method, the control of *Rhizoctonia solani* by *Verticillium biguttatum*, seems promising. Koppert has been investing with this project: research is carried out to develop the biocontrol agent into a product. As mentioned above there are still many problems to solve. The uncertainty about the profitability of the product and the registration demands makes the investments expensive and risky.

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# METHODOLOGICAL PITFALLS IN BIOCONTROL EXPERIMENTATION LEADING TO ERRONEOUS CONCLUSIONS

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## Introduction

In high throughput *in vivo* screens for microorganisms with the ability to control plant root diseases, the potential biocontrol agents (BCA) are normally tested as whole broths (cells plus spent growth medium), by application to plants inoculated with pathogen. Any significant protection of the plant from the pathogen is considered to be evidence for biological control, but this need not necessarily be the case. There are several drawbacks to screening microbial inocula as whole broths which can result in the recording of both false positives (apparent biological control where none exists) and false negatives (failure to detect biocontrol where it exists) in biocontrol screens. This paper will illustrate these problems and discuss ways of overcoming them.

## Results and Discussion

### 1. Phytotoxic, Nutritional and pH Effects Resulting from the Spent Growth Medium

#### a. Phytotoxic Effects

Some of the animal and plant extracts/digests commonly used in microbiological growth media are phytotoxic. Extrinsic phytoxicity of this nature (as opposed to the inherent phytotoxicity of a particular strain) could mask a potential biocontrol agent. It can be avoided by omitting the phytotoxic components from the inoculum growth medium, although a better solution is to use washed (eg. with phosphate buffer) inocula.

#### b. Nutritional Effects

Unutilised carbon sources in the spent growth medium of the inoculum can lead to inhibiton of plant growth by promoting the microbial immobilisation of plant growth nutrients such as nitrogen and phosphorus. This will have the effect of making a strain appear to be phytotoxic when it may not be inherently so and could mask a potential biocontrol agent. This problem should also be borne in mind when considering the use of food bases in formulations of biocontrol agents. Unutilised nitrogen sources in the spent growth medium of the inoculum can promote plant growth and make it less susceptible to disease, thereby giving the appearance of biocontrol. Microorganisms will vary considerably in the amounts of carbon and nitrogen they will remove from a particular medium, so some strains will give more striking effects than others. The problem of nutrient carryover is easily avoided by using washed inocula.

#### c. pH Effects

The final pH of a complex microbial growth medium at the end of a batch culture growth cycle can vary widely over the range pH 3-10, depending on the microorganism, if the medium is not particularly well buffered (which

applies to most media in common use). Plant growth nutrient solutions tend to have poor buffering capacities because of restrictions in the amounts of phosphate that can be tolerated by plants. Microbial broths at the extremes of the pH range quoted can shift the pH of plant growth nutrient solutions and thereby reduce plant growth and/or the activity of the pathogen. Reductions in plant growth can lead to biocontrol agents being missed and reductions in activity of the pathogen can be misinterpreted as biological control. The problem is best solved by using washed inocula, although an alternative would be to adjust all the broths to neutrality before screening.

2. Nutritional Effects Arising from the Biomass of the Inoculum

Microbial biomass is an excellent source of plant nutrients. Wheat can be grown to maturity in sand on a range of microorganisms as the sole source of mineral nutrition (Lethbridge and Davidson, 1983a). Microbial biomass nitrogen can be just as available as nitrate-nitrogen in some soils (Lethbridge and Davidson, 1983b). These experiments demonstrate that a significant portion of a microbial inoculum dies on application to sand or soil and undergoes rapid mineralisation by the native microflora, releasing mineral nutrients in a plant available form. Nutrition can have a profound effect on the extent of plant root diseases. A well nourished, fast growing plant is more likely to escape heavy infection than a poorly nourished plant. For this very reason many screens for biocontrol agents of plant root diseases are carried out at low nutrient levels to promote the activity of the pathogen.
How serious therefore is the risk of recording false positives in biocontrol screens resulting from enhanced plant growth at the expense of nutrients released from a dying microbial inoculum? The plant growth responses described above were achieved using 150-200 mg dry weight microbial biomass per plant. For the bacteria this was equivalent to approximately $10^{11}$ cells per plant. The cut-off point below which the inoculum size had no detectable effect on the yield or nitrogen content of wheat was found to be $10^{10}$ cells (15 mg dry wt) per plant (Lethbridge and Davidson, 1983a). Shake flask fermentations typically yield broths containing 1 - 10 mg dry weight microbial biomass per ml. For a unicellular bacterium this can represent between $10^8$ and $10^{10}$ cells per ml depending on the organism. Thus 1 ml of an undiluted broth at the upper limit is very close to the level at which the inoculum biomass can become a significant contributor to plant nutrition. Any greater volume at time zero, or in the form of repeated application rates at regular intervals over the duration of the experiment would certainly have a significant effect on plant growth and run the risk of recording a false positive in the biocontrol screen by promoting plant growth and thereby making it more resistant to infection by the pathogen. The best way round this potential problem is to ensure that the total amount of biomass in the inoculum never exceeds the level at which it becomes a significant contributor to plant nutrition.

3. Preformed Biologically Active Secondary Metabolites

The most serious pitfall in biocontrol screening occurs when secondary metabolites active against the pathogen under test are produced during the inoculum production fermentation, such that preformed bioactive molecules are applied to the screen along with the potential biocontrol agent. If the microorganism under test is not capable of producing the bioactive molecule in the root zone of the host plant, because its synthesis is not supported by

plant root exudate and the microorganism exhibits no other biocontrol properties, then biocontrol will be claimed where none exists. Even if the microorganism can synthesise the bioactive molecule in the root zone, or it posseses other biocontrol properties (eg. production of cell wall lytic enzymes, aggressive competitor) the screen is likely to over-estimate the true biocontrol potential of a microorganism tested as a broth containing a preformed bioactive product. The amount of product formation in a shake flask is likely to be considerably greater than in the root zone of a crop, since the amount of available carbon will be orders of magnitude greater in the former.

It is important to distinguish between a microbial inoculum acting as a true biological control agent and an inert delivery system for a biologically active natural product, because the latter is just a formulated chemical and as such is no different from any new synthetic, or natural product pure chemical. Consequently, the biologically active natural product should be identified and subjected to thorough toxicological and environmental testing to ensure its safety. In addition, the compound in question may already be under patent to an agrochemical, or more likely a pharmaceutical company and this so-called biological control product could well infringe such a patent. The pitfall of preformed bioactive product formation is not as easy to avoid as the other pitfalls discussed in this presentation. Harvesting the microbial biomass from the fermentation broth, followed by thorough washing prior to testing will only remove extracellular product. Many apolar metabolites are retained inside the biomass and even polar metabolites will not be found exclusively in the the broth filtrate or supernatant. Up to 10% w/w of a polar metabolite can remain inside the biomass. Intracellular metabolites are only released from the biomass upon cell death and this is how they become available to inhibit or kill the pathogen in a biocontrol screen.

Distinguishing between the contribution of true biological control and the activity of a preformed product to the total disease control observed in a screen is not always straightforward. Fractionation of the broth and subsequent testing of these fractions in the biocontrol screen can yield some useful information, but is not always conclusive. If uncertainties still exist after testing broth fractions, the next stage is to prepare an inoculum which lacks the preformed product. This can be done in the existing growth medium if product formation is not growth associated, ie. synthesis starts at the end of biomasss accretion phase. If product formation is growth associated then a range of alternative growth media formulated to achieve different physiological stresses (eg. carbon, nitrogen, phosphate and oxygen limitation) will have to be tried to find one in which product formation is suppressed. Any activity in the biocontrol screen elicited by washed biomass produced by either of these two methods is true biocontrol activity.

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# PROSPECTS FOR THE BIOCONTROL OF DISEASES OF CROPS GROWN IN BIOLOGICALLY-AMENDED SUBSTRATES

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## Summary

Plants are grown in a variety of substrates, ranging from composted organic refuse to high-tech man-made material. These plants are affected by a wide range of root-infecting diseases that may be susceptible to biological control. As well as the well-known benefits of biological control, there are a number of advantages afforded by the substrate for the biological control of substrate-grown crops. The initially sterile nature of many substrates would facilitate the production of axenic or gnotobiotic products. It may also allow the use of micro-organisms that have a low competitive saprophytic ability. The provision of a pre-treated substrate would reduce the time and money spent during the season by the grower introducing biocontrol agents. Prepared substrates are intrinsically less variable than field soils and are used in generally less variable environments, both features aiding the development of a reliable biocontrol strategy. Packaged substrates, annually replaced by the grower, would both reduce any accidental spread of the biocontrol agent and provide industry with a regular market for the treated substrate. The rhizosphere of substrate-grown crops is an ideal site for both the practice and study of biological control. The prospects are good for the biological control of diseases of substrate-grown crops using biologically-amended media.

## Types of substrate

The main manufactured substrates are those made from rock such as Perlite, Vermiculite and Rockfibre and organics like Polyurethane foam and Polyacrylamide gel. These relatively homogeneous products are sterile after manufacture and therefore contain few organisms with which any biocontrol agent inoculum has to compete. There is a range of natural substrates that normally have an initially low microbial content, i.e. Peat and Coir. These may be used either as soil amendments or as relatively inert growing media. Thirdly, there is a large number of composted organic products have been studied for their use as horticultural substrates, such as; sewage sludge and other organic by-products. These often have to be carefully prepared to remove toxic substances and may contain pathogen propagules within a high microbial population. Biological control may be practiced in any of these substrates. The media with a relativley low initial microbial content would be suited to beneficial biological amendment, while the organic composts may be naturally disease suppressive.

## Advantages and disadvantages of substrate-based biocontrol

The factors affecting choice, preparation and formulation of biological control agents for use against diseases of plants in substrates are generally similar to those for organisms to be applied to soil or other sites. However, there are advantages for the application and use of biocontrol agents in many substrates compared to their use in the rhizosphere of plants grown "in the field". The main advantage of the initially sterile substrates is that axenic or gnotobiotic products may be prepared. Although after sowing or planting, any substrate would gain a varied microflora, the initial lack of competitors in substrates may allow the use of microbial control agents with low saprophytic ability.

The use of a pre-treated substrate would remove the need for the grower to spend time and money applying the inoculum to the propagation site or during plant growth. The relatively homogeneous nature of substrates compared to the huge variation in natural soil types means that an agent developed and formulated for use in a particular substrate would be applicable for use in that substrate world-wide. This is particularly true for protected crops, where environmental conditions are relatively similar in greenhouses from one location to another. A range of biocontrol agents specific to particular substrates and environments as well as particular crops and diseases may be developed. The packaging of many commercially-used substrates such as peat bags or wrapped rockfibre slabs may also benefit a biocontrol programme. The packaged substrate, especially when used in a greenhouse, would reduce the perceived risk of accidental release of the biocontrol agent into the environment and facilitate the study of the distribution of the antagonist through the rhizosphere. Many packaged substrates are used to grow a crop for only one or two years, and are then disposed of. This has the benefit for the commercial biocontrol industry because the grower has to buy more product each season, unlike field situations where amended soil may exert its activity continuously after an initial treatment.

There are few disadvantages to practising biocontrol within substrates. The initial cost of the amended substrate may be a problem for the cash-flow of growers but should be countered by savings later in the season. Other potential problems with biological amendment of substrates are relevant to biocontrol in general: the use of a beneficial fungi, may preclude the application of fungicides to control fungal pathogens not affected by the antagonist, and improving plant health by reducing root disease may alter the plants susceptibility to other diseases.

Careful study of each disease/substrate situation is required for product development. There are many examples of useful antagonists of particular diseases having undesired affects on other diseases. For example a composted sewage sludge containing antagonists to certain species of *Aphanomyces, Rhizoctonia, Sclerotinia* and *Phytophthora,* actually stimulated some diseases caused by species of *Thielaviopsis* and *Phytium* (Lumsden *et al*., 1983). Similar results have been found with mycorrhiza. Recent work has shown that the symbiont *Glomus fasciculatum* was stimulated in the presence of the Poinsettia pathogen *Pythium ultimum* and disease was reduced (Kaye *et al.,* 1984). The same mycorrhiza had no effect on *Phytophthora cinnamomi* rot of citrus or alfalfa, but made the disease worse in avocado

(Davis *et al*., 1978). Research into the microbial ecology of root diseases may indicate methods of improving beneficial activity. It may be beneficial to inoculate substrates with 2 or more biocontrol agents. These may act synergistically against a particular disease, or against different diseases. Substrate type may affect the activity of the biocontrol agent. For example, the extent that *Glomus intraradices* reduced *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomatoes was dependent on substrate type; vermiculite, peat or sand (Caron *et al*., 1985). It may be possible, and necessary to develop a range of different microbial treatments for different crops, different crop ~~ages and~~ different environmental conditions. The use of certain organisms in amended substrates may require the grower to use a novel growing regime, and a compromise may be necessary between the optimum conditions for biocontrol activity and plant growth.

## Conclusions

If a substrate pre-treated with a biological control agent for a particular application is not developed, then it is probable that biocontrol will be practised in the substrate using a separate inoculum source. The advantages provided by substrates for biocontrol of plant diseases indicate that the prospects are good for producing reliable and economic biological control strategies of substrate-grown plants. This is even more likely with the current pressure from governments and environmental groups to increase the re-use of certain substrates and reduce the application and waste of pesticides.

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INDUSTRIAL-ACADEME PARTNERSHIP: A CRITICAL REQUIREMENT FOR THE DEVELOPMENT OF BIOCONTROL SYSTEMS

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Summary

Biological control systems frequently require corporate-academic cooperation if they are to be commercially useful. The initial discovery of an effective organism, genetic manipulation of organisms to make them more effective, and studies on mechanisms usually are carried out in academe. Corporate resources are required for large-scale production, toxicology, wide-scale field testing, registration, and marketing. Many other aspects, especially production methods, formulation, and delivery systems may fall clearly into neither academic or corporate responsibility. Moreover, development and integration of this intermediate technology frequently is time-consuming and is an area for which few people are trained. Corporate funding and assistance are essential for this mid-level development. In order for an academic-industry team to be established, entrepreneurship is required of the academic research team. Entrepreneurship may be defined as the exchange of intellectual property for research grant dollars and a royalty stream, with the establishment of a university-industry partnership for the benefit of both. The first requirement for entrepreneurship in biocontrol is a patent application on a strain and related technology; usually substantial efficacy data and information on commercially-useful delivery systems are required. Then the academic research team can begin "corporate courting", i.e. distribution by mail of information on the technology that is available, followed by personal selling visits to interested companies. Once general agreement between the corporation and the university has been agreed upon, a contract must be prepared. Very important issues, including confidentiality, prepublication review, distribution of strains, diligence in development of technology, the nature and length of rights to intellectual property, must be precisely defined. These negotiations will always contain a degree of negotiation and tension, since corporate interests and university tradition may be at odds. For example, it usually is in corporate interest to retain research results as confidential, proprietary information, while universities regard openness and dissemination of information as critical to the academic process. Ideally, the process of entrepreneurship will result in an academic-corporate research team working towards a common goal. The desirability of building such a team and impediments to achieving this will be discussed.

# Regulations for and experience with the registration of biological pesticides in the Netherlands

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## 1. Definition of biological pesticides

In the Netherlands pesticides have to be registered before they may be used, kept and sold. Biological pesticides fall within the Pesticide Act.
In the legislation products based on micro-organisms (fungi, bacteria) and viruses are called biological pesticides.
Genetically manipulated organisms fall within a Special Act.
Nematodes, predators and insect parasites are exempted from registration.
Feromones and toxines are considered as chemical pesticides.

## 2. Registration procedure

In the Netherlands four Ministries are responsible for the registration of (biological) pesticides:

* Ministry of Agriculture, Nature Management and Fisheries
  - efficacy and composition
* Ministry of Welfare, Public Health and Cultural Affairs
  - health aspects
* Ministry of Housing, Physical Planning and Environment
  - safety for the environment
* Ministry of Social Affairs and Employment
  - labour safety for the user

The decisions of these Ministries are coordinated by the Committee for Pesticide Registration. This Committee is an official body of which the members represent their ministries. The Committee prepares advices about the regulations of pesticides to the responsible Ministers.
For the registration of biological pesticides a questionnaire has been drafted. The lay-out of the data sheets corresponds with the advice and recommendation of the Council of Europe.
At present the draft questionnaire is seen within the light of the Council directive of the European Community concerning the placing of pesticides on the market. This directive consists of procedures and executive guidelines (so called uniform principles).
This questionnaire is divided in eight chapters:

A: General information.
B: Information on application.
C: Information on formulated product.
D: Information on active agent(s).
E: Information on toxicity of the product, active agent(s) and metabolites.
F: Information on residues.
G: Behaviour in soil, water and air.
H: Toxicity to naturally occuring organisms.

## 3. History of biological pesticides in the Netherlands

Biological pesticides are not new. In the Netherlands the first biological pesticide was registered in 1971. This product was based on

the bacterium *Bacillus thuringiensis* for the control of lepidopterous larvae in cabbage, orchards and forestry. Up to now seven products based on *Bacillus thuringiensis* have been registered and for a few approval is pending with the Committee for Pesticide Registration. These products are used against the wax moth in bee hives, lepidopterous larvae in cabbage, orchards, forestry and fruiting vegetables and *Diptera* in cavities of houses.

The history of other biological pesticides is brief. The first biological pesticide based on a fungus was submitted for approval ten years ago, however rejected, because its efficacy was insufficient. At present three biological pesticides based on fungi (against white fly in cucumbers, Dutch elm disease, and *Fusarium* footrot in tomatoes on rockwool) and one based on a baculovirus (codling moth) have been submitted for approval in the Netherlands.

## 4. Evaluation of the composition and efficacy

One of the main responsibilities of the Plant Protection Service (Ministry of Agriculture, Nature Management and Fisheries) is evaluating the composition and efficacy of agricultural pesticides. The procedure for biological pesticides has four steps:

1. Evaluation of the information submitted in the request.
2. Examination of the composition of the product.
3. Supervision of the field trials on efficacy.
4. Evaluation of the agricultural efficacy.

For the evaluation of the information on the application form the answers in the chapters A till D are studied.
The applicant could be questioned additionally.
The check of the composition is carried out in the laboratory of the Plant Protection Service. As a matter of course biological methods have to be used. This verification of the composition has two aspects:

a. The identity of the active agent(s).
b. The content of active agent(s) in biological active units.

Verification of the identity of the active agent(s) is to check whether the organism(s) given by the applicant is present.
Different methods of identification are used e.g. morphological or biochemical identification, spectrum of activity.
Verification of the content of the active agent(s) is necessary to check whether this content (in biological active units) given by the applicant is correct.
The method of analysis has to be submitted by the applicant. Methods for determining the content of biological pesticides can be divided in two categories:

- counting (fungi)
- bio-assay (*Bacillus thuringiensis*, baculoviruses).

Field trials are carried out by the manufacturer and are supervised by experts of the Plant Protection Service. These trials have to be in accordance with the EPPO guidelines of chemical pesticides or other guidelines approved by the Plant Protection Service.
The efficacy data are evaluated by the Plant Protection Service.
Efficacy is the ability to fulfill the claims made for it on the label.

The general criterion for efficacy is:
- satisfactory levels of efficacy will be met when the performance is comparable to that of a suitable standard product.

In some cases the use of a reference product is inappropriate or impossible. In that case the product should show a well defined benefit to the user and should be able to bring the damage below the economic threshold level.

For biological agents the next criterion can be used:
- Adventages of the biological pesticide for the environment, the consumer and the user may compensate deficiences in e.g. level or consistency of efficacy.

The opinion about the agricultural efficacy is discussed in the Interdepartemental Committee that advices on the registration of the pesticides.

5. Experiences

During the registration procedure of some biological agents a number of specific examination aspects of biological pesticides emerge.

5.1. Composition

- Formulation

The formulation of biological pesticides is not always satisfactorily to get a homogenous spraying liquid. In some cases the active ingredient settles to fast to the bottom of the spray-tank because of an insufficient formulation of the product or because of the presence of too large particles for instance spore clots.

The determination of the content causes problems in some formulations of biological pesticides. For instance a formulation with many spore clots shows a considerable spread of the results of the analysis of the number of colony forming units.

- Standardization.

The content of some products is examined by means of a bio-assay with a certain testinsect. The biological activity of a product is compared to that of a standard product. Therefore some International Standard preparations have been developed for instance for *Bacillus thuringiensis* and the granulosis virus of the codling moth.

To compare products based on the same agent of various manufacturers standardization of the methods of bio-assay is necessary. For instance for products based on *Bacillus thuringiensis subsp. kurstaki* different manufacturers use different methods with different test insects. Some manufacturers use *Anagasta kühniella* and others *Trichloplusia ni.* Comparing the content of those products is therefore impossible.

- Shelf-life.
  The shelf-life of biological products varies from 2 months at 4°C (e.g. suspensions based on fungal spores) to 2 years at room temperature (e.g. powders based on _Bacillus thuringiensis_). The duration of the application period determines the minimal shelf-life.

5.2. _Field experiments_

At present there are no EPPO guidelines for carrying out field experiments with biological pesticides. The design of the trials differ from the guidelines of field tests with chemical pesticides because of the nature of biological agents.
The use of a reference product is not always possible in the field test for instance when the test product is a part of an integrated pest management system.

5.3. _Application aspects_

The application of biological pesticides deserves special attention. For instance:

- application time. The spores of _Verticillium lecanii_ need a period of high relative humidity to germinate and to penetrate the host. The application should take place in the evening.
- Compatability with chemical pesticides.
- Safety for other crops for instance _Chondrostereum purpureum_ as a biological agent for the control of Prunus serotina in the forest is also pathogenic to plum-trees and apple-trees.
- Special application techniques.

From this review it appears that the registration procedure of biological control agents is a developing process. Nowadays a lot of research for biological agents takes place and it is to be expected that an increasing number of agents will be developed to biological pesticides. Every biological pesticide submitted for approval is evaluated individually, because the requirements have not been clear sofar and every agent has its own characteristics and often special application techniques.
In the future it is important to establish international harmonization of registration requirements and cooperation with the standardization of test methods (e.g. EPPO guidelines).
Finally fruitful cooperation between research, industry and authorities is necessary to stimulate the development, registration and use of biological pesticides.